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The Catalysis of the Auto-oxidation of 2-Mercaptoethanol and other Thiols by Vitamin B₁₂ Derivatives

POLAROGRAPHIC AND OTHER INVESTIGATIONS

By J. L. PEEL

Agricultural Research Council Unit for Microbiology, The University, Sheffield 10

(Received 7 January 1963)

During a study of the exchange reaction between CO₂ and the carboxyl group of pyruvic acid catalysed by preparations from *Peptostreptococcus elsdenii* (previously referred to as rumen organism LC), it was found that the auto-oxidation of 2-mercaptoethanol (monothioethylene glycol) is greatly accelerated by catalytic amounts of vitamin B₁₂ derivatives at pH 7 (Peel, 1962*a*). Such an effect does not appear to have been reported previously at physiological pH, except for an incidental statement by Dubnoff (1950) that vitamin B₁₂ catalysed the oxidation of certain thiols in a non-enzymic system. In view of the current interest in vitamin B₁₂ derivatives, the increasing use of 2-mercaptoethanol as a reducing agent in incubation mixtures and the possibility that this catalytic property might be of use for the detection and assay of vitamin B₁₂ derivatives, the reaction was investigated further. The present paper reports the results, some of which have appeared in brief (Peel, 1962*b*).

MATERIALS AND METHODS

Chemical reagents

The thiols used were as follows: 2-mercaptoethanol, Eastman grade (from Kodak Ltd., Liverpool); 2,3-dimercaptopropanol (BAL) and L-cysteine hydrochloride (from L. Light and Co. Ltd.); thioglycolic acid, laboratory-reagent grade (from British Drug Houses Ltd.); glutathione [from The Distillers Co. (Biochemicals) Ltd., Liverpool]; DL-dihydro-6-thioctic acid, assay 98%, and DL-dihydro-6-thioctic amide, assay 100% (from Farmochimica Cutolo-Calosi S.p.A., Naples; gifts from Dr V. Massey). The 2-mercaptoethanol, 2,3-dimercaptopropanol and thioglycolic acid were distilled under reduced pressure before use and then assayed to be 100, 100 and 95% pure respec-

tively by iodometric determination. The 2-mercaptoethanol was subsequently used over an extended period during which it was stored at room temperature. After 6 months the purity had fallen to 92% and the material was distilled again. In experiments where the amount of thiol was critical, solutions were standardized iodometrically before use. Where necessary thiol solutions were neutralized to pH 7.1 with NaOH, immediately before use. Aqueous 50% (v/v) methanol was used to dissolve the dihydrothioctic acid amide. Controls showed that this solvent had no effect on the polarographic measurements.

The vitamin B₁₂ derivatives were obtained as gifts and, apart from the exceptions mentioned below, were from Dr E. Lester Smith. Their structural relationships to cyanocobalamin are indicated in Table 3. The 5,6-dimethylbenzimidazolylcobamide coenzyme (Barker *et al.* 1960*b*) was obtained from Dr H. A. Barker. As the cobamide coenzymes are very labile to light, experiments with this compound were done in near darkness with minimum artificial illumination. Factors A and B (Ford & Porter, 1953) and Factor D (Brown, Cain, Gant, Parker & Smith, 1955) were obtained as solutions in aqueous 70% (v/v) ethanol from Dr J. W. G. Porter and Dr H. R. V. Arnstein. These solutions were standardized by measurement of the extinction at 367 mμ in 0.1 M-KCN and assuming ε to be 30.4 × 10³ l. mole⁻¹ cm.⁻¹ as for dicyanocobalamin (Barker *et al.* 1960*a*). The term Factor B is usually used to denote the monocyano derivative of cobinamide, derived from cyanocobalamin by removal of the nucleotide, but as this compound readily takes up an additional molecule of cyanide, the scope of the term has sometimes been extended to include the dicyano form as well. The spectrum of the Factor B sample used in all the experiments reported below corresponded to a mixture of the mono- and di-cyano forms. On adding excess of cyanide, the spectrum changed to one closely resembling that for dicyanocobinamide given by Ford & Porter (1953). Factor B has not yet been crystallized; as far as the author is aware, reliable extinction coefficients, which would permit an evaluation of the proportions of the two forms in the mixture, are not available. The

extinctions of the sample at pH 7.1 in 5 mm.-potassium phosphate buffer at 367 and 580 m μ (where dicyanocobinamide has absorption maxima) were 86 and 73% respectively of the values in the presence of 0.1 M-KCN. For convenience, this preparation is referred to as 'Factor B' throughout. A second sample, which spectroscopic examination showed to be in the monocyano form, was used in experiments already published (Peel, 1962*a*). This sample was exhausted before its catalytic activity could be compared quantitatively with the other sample, but no qualitative differences in behaviour were noticed.

The remaining chemicals were of analytical-reagent grade except the following: ascorbic acid B.P. (from Hopkin and Williams Ltd.); disodium ethylenediaminetetraacetate, Na₂S₂O₄, Na₂H₂P₂O₇, NaN₃ and 1,10-phenanthroline monohydrate (from British Drug Houses Ltd.); and Fe₂(SO₄)₃, 'pure' (from Towers and Co.).

Extract of boiled yeast

This was prepared by mixing baker's yeast with water (2 ml./g. of yeast), heating at 100° for 10 min. and then removing insoluble material by centrifuging for 20 min. at 10 000 g. The extract contained about 30 mg. dry wt./ml. and, where indicated, was freeze-dried.

Determination of thiols

Aqueous solutions of thiols were standardized iodometrically by the method described by Woodward & Fry (1932) for glutathione, but with the following modifications. The preliminary deproteinization was omitted, the titration was done in the presence of 0.1 N-H₂SO₄ in place of sulphosalicylic acid, and 1 mM-KIO₃ was used. For determinations on the products from manometric experiments, the manometer contents plus washings were transferred into 10 ml. of 0.2 N-H₂SO₄ and titrated without adding further acid.

Spectrophotometric measurements

Extinctions were measured with the Unicam SP. 500 or SP. 600 instruments. Spectra were scanned with an Optica recording spectrophotometer [Optica (U.K.) Ltd., Gateshead-on-Tyne].

Manometric measurement of thiol oxidation

This was done in Warburg manometers at 37° and with air as gas phase by conventional techniques.

Polarographic measurement of thiol oxidation

Apparatus. The oxygen uptake due to thiol oxidation was also measured by following the diminution in the (partial) pressure of oxygen polarographically. The oxygen electrode used was of the Clark (1956) type, the advantages of which have been pointed out in a review by Connolly (1957). The essential feature of the design is that the Pt/oxygen electrode, together with the Ag/AgCl reference electrode and the connecting electrolyte, are enclosed by a thin polyethylene membrane, readily permeable to gases but not to other solutes. This membrane shields the oxygen electrode from substances in the test solution, e.g. protein and precipitating ions, that would otherwise inactivate the electrode. Apparatus incorporating such electrodes, suitable for the measurement of respiration, has been described by Bellamy & Bartley (1960) and by Stickland (1960). The

current flowing at a given (partial) pressure of oxygen is increased by movement of the test solution past the membrane and in the procedure used by the above authors the oxygen pressure cannot be measured until the transient disturbance associated with the addition of the last component to the reaction mixture has subsided. In the writer's experiments this difficulty was overcome by stirring the reaction mixture continuously at a constant rate.

The apparatus was designed to present the minimum liquid surface to the outside air and its construction is shown in Fig. 1. It consisted of a cylindrical glass reaction chamber, *RC*, surrounded by a water jacket, *J*, through which water of controlled temperature was circulated by means of a Tecam Tempunit [Technique (Cambridge) Ltd.]. The contents of the reaction chamber were stirred magnetically, the stirrer slug, *S*, being made from a piece of mild-steel rod (12 mm. long $\times$ 3 mm. diam.) sealed in a short section of polyethylene tube (external dimensions: 18 mm. long $\times$ 5 mm. diam.). The jacketed vessel was centred over a magnet, *RM*, which rotated at about 250 rev./min. A Perspex plunger, *P*, fitted loosely into the cylindrical reaction

Fig. 1. Apparatus for polarographic measurement of thiol oxidation. *RC*, Reaction chamber; *J*, constant-temperature water jacket; *S*, magnetic stirrer slug; *RM*, rotating magnet; *P*, Perspex plunger; *SR*, supporting rod; *PS*, polyethylene sleeve; *G*, vertical groove; *E*, combined electrode assembly; *Ag*, silver/silver chloride anode; *Pt*, platinum cathode; *PM*, polyethylene membrane; *C*, cable leading to electrical circuit. For simplicity, much of the interior detail of the combined electrode assembly has been omitted.

vessel leaving an annular gap of about 0.5 mm. width. To this was attached a stainless-steel supporting rod, *SR*, which passed through a clamp above the chamber and was used to raise or lower the plunger. A combined electrode assembly, *E*, manufactured by the Yellow Springs Co. Inc., Yellow Springs, Ohio, U.S.A., projected through the plunger and was held in position by a tight-fitting polyethylene sleeve, *PS*. The polyethylene membrane, *PM*, was cut from a sheet of mean thickness 0.002 in. The lower surface of the plunger was inclined at an angle of 5° to the horizontal and from the highest point a V-shaped vertical groove, *G*, measuring about 1.5 mm. from apex to circumference, led to the upper surface of the plunger. When the plunger was lowered on to the surface of a reaction mixture any air bubbles escaped through this groove so that the only liquid surfaces remaining in contact with the air were those in the groove and the annular gap. An adjustable stop was fitted to the supporting rod above its clamp and was set so that with 8 ml. of liquid in the reaction chamber and the plunger lowered, the liquid surface came halfway up the vertical groove. After use, the plunger was raised and the reaction chamber emptied by suction from a water pump. The plunger and chamber were then rinsed with water, the rinsings were sucked away, and the electrode was blotted dry with paper tissue.

The combined electrode was connected by the cable, *C*, to a polarographic circuit like that of Stickland (1960), except that the condensers across the batteries were omitted. The voltage applied across the combined electrode was 0.6 v. The galvanometer was a Scalamp model (W. G. Pye, Cambridge) and a 560 Ω shunt was placed across it to give a convenient sensitivity. All measurements were then made with the sensitivity set to '1', and under these conditions a scale deflexion of 9.4 cm./μA was obtained.

Calibration. The expected relationship between electrode current and oxygen concentration was a linear one (manufacturer's manual; cf. Bellamy & Bartley, 1960) and this was verified by measuring the current flowing when the test solution was saturated with a series of air-N₂ mixtures of known composition. For routine calibration, the current at air saturation and at zero oxygen concentration was measured. The upper calibration point was determined by putting into the reaction chamber 7.7 ml. of the appropriate buffer, raising the plunger to give about 4 ml. of air above the liquid, and increasing the rate of stirring so that a wide vortex formed. After 3–5 min. the stirrer speed was decreased to normal and the plunger restored to the fully lowered position. The galvanometer reading was then noted as soon as it became steady. This aeration process was repeated until the galvanometer reading was constant. The 'residual current', i.e. that flowing at zero oxygen pressure, was determined by adding a few milligrams of sodium dithionite to the air-saturated buffer and noting the reading after 2–3 min. This was found to be identical with that observed when the buffer was saturated with N₂ (O₂-free). Saturation with N₂ or with air-N₂ mixtures was achieved in a similar fashion to air saturation except that a Pasteur pipette was inserted through the groove, *G*, into the liquid, and gas of the appropriate composition was passed.

The term 'oxygen current' is used below to denote the quantity: (observed current – residual current). The corresponding galvanometer deflexion was directly proportional to oxygen concentration for a given buffer solution and for many purposes it was sufficient to express results in

terms of this corrected galvanometer reading. Where absolute values of oxygen concentration were required, it was assumed that, during determination of the upper calibration point, the air in equilibrium with the test solution was saturated with water vapour. The concentration of dissolved oxygen was then calculated from the relationship:

$$C = \alpha \times 1000 \frac{(p_a - p_w)}{760} \times 0.21$$

where *C* is the oxygen concentration in μl./ml., α is the absorption coefficient for oxygen, *p_a* is the atmospheric pressure, and *p_w* is the saturation vapour pressure of water in mm. Hg; 0.21 is the fraction by volume of oxygen in air.

The oxygen current with the Clark electrode appears to be determined by the partial pressure rather than the concentration of oxygen in solution since the upper calibration points were identical for water, 0.1 M-phosphate buffer and for 5% (w/v) NaCl, in which oxygen is considerably less soluble than in water (e.g. approx. 25% less at 30°). The presence of salts thus affects the calibration of the instrument in terms of oxygen concentration. In the absence of solubility data for the appropriate buffers, their effect on oxygen solubility was ignored in calculating rates of oxygen uptake. Data from standard tables for other solutes suggest that the error so introduced is probably less than 10% with 0.1 M-phosphate buffer.

Both residual current and oxygen current increased with temperature and it was necessary to calibrate the instrument separately at each temperature. There was no significant effect of pH on the calibration within the pH range used (5.5–9.0). Although the response of the electrode was increased by stirring, stirring rates greater than normal (250 rev./min.) did not give a higher current. The oxygen current was dependent on the polyethylene membrane, and for air-saturation ranged from 1 to 1.25 μA with different samples of the same polyethylene sheet. It was therefore essential to recalibrate after changing the membrane.

When temperature, stirring and membrane remained unchanged the calibration points still underwent certain variations. Transient high currents were always observed when the electrical circuit was switched on. After 5–15 min. readings became steady in the sense that changes over the period required for each measurement of thiol oxidation were negligible. The following remarks refer to currents after this initial 'warming up' period. The oxygen current remained constant apart from small slow fluctuations attributable to changes in atmospheric pressure. The residual current was subject to long-term changes that appeared to be related to the state of the electrolyte and the membrane. When both of these had been newly changed the residual current was high (as much as 1 μA) but fell to a value less than 0.1 μA after a few hours' running. After this 'running in' period it remained constant but finite for a lengthy period (e.g. 100 hr.). Eventually the residual current began to rise and the upper calibration point moved beyond the galvanometer range. This effect could be offset by applying a 'backing off' current to the galvanometer as described by Stickland (1960), but this remedy was only used to avoid breaking off during a series of experimental runs. A low residual current could usually be restored by changing the KCl electrolyte and 'running in' afresh; when this failed, changing of the membrane was effective. The long-term rise in residual current was believed to be due to slow contamination of the electrolyte or the membrane.

There were some indications that prolonged exposure to dithionite solution could produce this effect and care was therefore taken to clean out the apparatus as soon as possible after determination of the lower calibration point.

Operation. For measurements of thiol oxidation the procedure was as follows, except for the modifications indicated in individual experiments. The apparatus was switched on at least 30 min. before it was required. Meanwhile 0.1M-phosphate buffer, pH 7.1 (KH₂PO₄-KOH mixture), was equilibrated with air at 37° by shaking in a shallow layer. The apparatus was calibrated and 7.7 ml. of buffer was placed in the reaction chamber. With the plunger lowered and the stirrer running, the apparatus was left until the galvanometer became steady as thermal equilibrium was attained (3–5 min.). The plunger was then raised and the stirrer stopped temporarily while 0.3 ml. of 0.8M-2-mercaptoethanol was introduced. When the plunger was lowered again all air was displaced from beneath. Readings were taken over the next 5 min., by which time a slow blank rate of oxygen uptake was established. The sample of vitamin B₁₂ derivative, in a volume not exceeding 0.1 ml., was next introduced through the vertical groove to start the reaction. Galvanometer readings were taken at suitable intervals (usually every 10 sec.) for the next 3 min. or until the oxygen concentration fell to zero.

The vitamin B₁₂ derivative was added from a 0.1 ml. pipette, the end of which was extended by a short length of flexible nylon tube (1 mm. bore) (catalogue no. 3A; Portland Plastics Ltd., Hythe, Kent) which was inserted through the vertical groove. In preliminary experiments the sample was added from a micrometer syringe but this method was abandoned after a period of erratic results that were later attributed to interference by metal ions derived from new syringe needles. In calculating the final concentration of substances in the reaction mixture it has been assumed for convenience that they become distributed uniformly throughout a reaction volume of 8.0 ml. When substances other than those mentioned above were present, they were added in small volume with the buffer and the volume of buffer was decreased accordingly.

The changes in oxygen concentration in the experiments described below were more rapid than those previously measured with the Clark type of electrode (e.g. by Bellamy & Bartley, 1960). With the present apparatus, the maximum rate that could be accurately followed was set by the ability of the operator to follow the galvanometer and was about 5 μ l. of O₂/ml./min. (12 cm. change in galvanometer reading/min.). The speed of response of the combined electrode was not a limiting factor, since the linear dependence between the rate of change in galvanometer reading and catalyst concentration held up to the above maximum (Fig. 5). Further, on adding excess of Factor B as catalyst, a rate of approx. 40 cm./min. was obtained and this limit appeared to be set by the inertia of the galvanometer.

RESULTS

Manometric measurement of the oxidation of 2-mercaptoethanol

When 2-mercaptoethanol was incubated in air at 37° in phosphate buffer, pH 7.0, an uptake of between 30 and 60 μ l./hr. was observed. When 35 μ M-Factor B was added to the system there was

a much more rapid uptake of gas (Fig. 2). The action of the Factor B is clearly catalytic and in some experiments a significant stimulation was observed with concentrations as low as 1 μ M. Cyanocobalamin and hydroxocobalamin also stimulated the reaction, though much larger concentrations (5 and 0.5 μ M) were required to produce a comparable rate (Fig. 2). The progress curves were not linear and there were indications that this was due to unknown reactions of the vitamin B₁₂ derivatives with mercaptoethanol which altered their catalytic activity. Light appeared to be unimportant.

The gas uptake was believed to be due to the oxidation of the mercaptoethanol to the disulphide form as follows:

This was checked by following the reaction with 50 mM-mercaptoethanol and 35 μ M-Factor B until the oxygen uptake had almost ceased. The total oxygen uptake at this point was only 85 % of that required for complete oxidation of the thiol initially added, but iodometric measurements showed that some thiol remained (Table 1). When the residual thiol was taken into account, the observed oxygen uptake was 96 % of that expected from the amount of thiol disappearing.

Fig. 2. Effect of vitamin B₁₂ derivatives on the oxidation of 2-mercaptoethanol, measured manometrically. Manometers contained, in 2.0 ml.: 0.22M-phosphate buffer, pH 7.0 (KH₂PO₄-NaOH mixture), 0.2M-mercaptoethanol and vitamin B₁₂ derivative as indicated. The last-named was initially placed in the side arm in 0.1 ml. of 0.2M-phosphate buffer and tipped after 20–30 min. of equilibration. The temperature was 37°; the gas phase was air. ○, No vitamin B₁₂ derivative; ●, 35 μ M-Factor B; △, 0.5 μ M-hydroxocobalamin; ▲, 5 μ M-cyanocobalamin.

Polarographic measurement of the oxidation of thiols

Decrease in oxygen concentration in the presence of mercaptoethanol and vitamin B₁₂ derivatives. For further study of thiol oxidation, the polarographic method proved to be more suitable than manometry. The reaction could be started by introducing the vitamin B₁₂ derivative into the reaction vessel, adequate measurements could be obtained within the ensuing 3 min. and lower thiol concentrations could be used. These factors were expected to diminish any effects due to modification of the catalyst. Progress curves with 30 mM-mercaptoethanol and Factor B, hydroxocobalamin or cyanocobalamin as catalyst are given in Fig. 3. In each case the oxygen concentration fell linearly with time until it reached about one-fifth of the initial value, after which the rate decreased progressively as zero oxygen concentration was approached. The final concentration corresponded to an oxygen pressure of less than 2 mm. Hg. A slow but consistent consumption of oxygen was observed in the absence of any vitamin B₁₂ derivative. With a few exceptions, specifically mentioned, all of the catalytic substances investigated gave similar progress curves. Rates were measured over the linear phase and duplicate measurements agreed within 5%.

Effect of pH. The effect of pH on the rate of the reaction was investigated within the pH range 5-9 by using citrate-phosphate, phosphate and pyrophosphate buffers. At pH less than 6 the rate was very low, but at pH greater than 6.5 it increased rapidly, reaching a plateau at pH 8.0 (Fig. 4). This pH-dependence does not reflect titration of the mercaptoethanol as the determined pK for this compound was about pH 9.5. The maximum rate may depend on the nature of the buffer since with glycine-sodium hydroxide buffer at pH 8.7 the rate was about half of that with pyrophosphate. The blank rate in the absence of catalyst rose markedly towards pH 9, making it impossible to investigate the rate with catalyst at higher pH values. Subsequent rate measurements were made at pH 7.1

Table 1. Stoichiometry of the oxidation of 2-mercaptoethanol

Experimental conditions were as for Fig. 2 except that the indicated amount of mercaptoethanol was added as a freshly standardized solution immediately before assembling the manometers. The catalyst was 35 μ M-Factor B. The reaction time was 200 min.

	Amounts (μ moles)	
	With Factor B	Without Factor B
Mercaptoethanol (initial)	110.4	110.4
Mercaptoethanol (final)	12.0	85.5
Mercaptoethanol consumed (a)	98.4	24.9
Theoretical O ₂ uptake (a/4)	24.6	6.2
Observed O ₂ uptake	23.5	5.8

Fig. 3. Effect of vitamin B₁₂ derivatives on the oxidation of 2-mercaptoethanol, measured polarographically. The reaction mixture initially contained, in 8.0 ml.: 95 mM-phosphate buffer, pH 7.1 (KH₂PO₄-KOH mixture), and 30 mM-mercaptoethanol. The vitamin B₁₂ derivatives, in 0.1 ml. of 0.2 M-phosphate buffer, were added at zero time. Oxygen concentration is expressed in terms of the corresponding galvanometer deflection (corrected for residual current) to which it is directly proportional. 1 cm. $\equiv$ 0.106 μ A $\equiv$ 0.41 μ l. of O₂/ml., except with CoSO₄ where 1 cm. $\equiv$ 0.106 μ A $\equiv$ 0.48 μ l. of O₂/ml. Other details are given in the Materials and Methods section. $\circ$, No vitamin B₁₂ derivative; $\bullet$, 9 μ M-Factor B; $\triangle$, 2.8 μ M-hydroxocobalamin; $\blacktriangle$, 30 μ M-cyanocobalamin; $\square$, 10 μ M-CoSO₄. For clarity, the curves for hydroxocobalamin, cyanocobalamin and CoSO₄ have been displaced 0.5, 1 and 2 min. to the right respectively, and intermediate experimental points on the curves have been omitted.

Fig. 4. Effect of pH on the oxidation of 2-mercaptoethanol. Details were as for Fig. 3 except that 8.0 ml. contained 7.7 ml. of buffer at the pH indicated. The catalyst was approx. 5 μ M-Factor B. The buffers were: $\circ$, 50 mM-citric acid-0.1 M-Na₂HPO₄ mixtures; $\triangle$, 0.1 M-phosphate (KH₂PO₄-KOH mixtures); $\square$, 0.1 M-pyrophosphate (Na₂H₂P₂O₇-NaOH mixtures); $\bullet$, $\blacktriangle$ and $\blacksquare$ indicate the corresponding blank rates in the absence of Factor B. The rates with Factor B have been corrected by subtraction of these blank values. The oxidation rate is expressed as the rate of decrease in galvanometer deflection in cm./min. 1 cm. $\equiv$ 0.48 μ l. of O₂/ml.

in phosphate buffer. This choice is a compromise: the oxidation rate is only about half the maximum, but the blank rate in the absence of catalyst is small at this pH.

Effect of temperature. With Factor B as catalyst the rates of oxygen consumption at 25° and 30° were 27 and 52 % respectively of that at 37°.

Effect of mercaptoethanol concentration. The relationship between rate and thiol concentration up to 150 mM showed a saturation effect similar to that with enzyme-catalysed reactions, the concentration of the thiol giving half-maximal velocity being 30 mM. This concentration was chosen for routine measurements on the same grounds as those used in selecting pH. The blank rate without catalyst was directly proportional to thiol concentration (0.55 cm./min. $\equiv$ approx. 2 μ l. of oxygen/min. at 150 mM).

Effect of catalyst concentration. With Factor B, the rate was directly proportional to the amount of catalyst present up to the maximum rate that could be accurately followed with the apparatus (Fig. 5). A similar relationship was observed with cyanocobalamin and hydroxocobalamin. Measurements for the other vitamin B₁₂ derivatives were limited, but each derivative was tested at more than one concentration and gave results consistent with a linear relationship.

Reaction with other thiols. A number of other thiols were compared with mercaptoethanol, Factor B being used as catalyst. These tests were

Fig. 5. Effect of Factor B concentration on the oxidation of 2-mercaptoethanol. The indicated volumes of approx. 1.8 μ M-Factor B in 0.2M-potassium phosphate buffer, pH 7.1, were added at zero time. Other details were as for Fig. 3. The oxidation rate is expressed as in Fig. 4. 1 cm. $\equiv$ 0.48 μ l. of O₂/ml.

carried out with 30 mM-thiol, except where this gave inconvenient rates. The reaction was only followed to completion with 2,3-dimercaptopropanol and the thioctic acid derivatives, but, with this proviso, all progress curves were similar in form to those with mercaptoethanol. Factor B caused a marked stimulation in all cases, the increase being several times the blank rate in its absence, except with 2,3-dimercaptopropanol (Table 2). Despite redistillation, this compound gave a considerable blank, and the effect of Factor B with a thiol concentration of 30 mM could not be tested. With thio-glycollate, cysteine or glutathione at a concentration of 30 mM, the rate due to Factor B was considerably less than with mercaptoethanol. The reduced forms of 6-thioctic acid and of its amide were much more active than mercaptoethanol, concentrations of 2.5 and 1.25 mM respectively giving the same rate as 30 mM-mercaptoethanol. One non-thiol, namely ascorbic acid, was tested for comparison, and no catalysis by Factor B was detectable, although a rapid oxidation resulted on adding Cu²⁺ ions (cf. Meiklejohn & Stewart, 1941). Mercaptoethanol appeared to be the best thiol for general use, as the thioctic acid derivatives, although more active, were only available in limited amounts and are not readily soluble in water.

Activities of other vitamin B₁₂ derivatives. A number of vitamin B₁₂ derivatives were tested with mercaptoethanol. All proved to be active and with one exception gave progress curves of similar form to that with Factor B. The exception was Factor A, with which the rate increased progressively during the first half of the reaction. The maximum rate was used to calculate the activity of this compound. Since, in all cases examined, the rate was directly proportional to catalyst concentration, activities are expressed as μ l. of oxygen uptake/min./ μ m-mole of catalyst and are given in Table 3, together with structural details.

Of the compounds tested, the least active was the dimethylbenzimidazolylcobamide coenzyme (no. 2 in Table 3) with one-third of the activity of cyanocobalamin (no. 1). In these two compounds, 5'-deoxyadenosine and cyanide respectively are linked to the cobalt atom; hydroxocobalamin (no. 3), which lacks these substituents, was ten times as active as cyanocobalamin. Analogues of cyanocobalamin with modifications in the short side chains attached to the corrin nucleus (nos. 4-10) or in the base of the nucleotide moiety (no. 11) were up to 50 times as active. Factor B (no. 12), which differs from cyanocobalamin in containing no nucleotide, was approx. 4000 times as active (the value in Table 3 is a revision of that quoted by Peel, 1962b). Factor D (no. 13) was the only other derivative tested that had an activity approaching that of Factor B.

Table 2. *Rates of oxidation with different thiols*

Reaction mixtures were as for Fig. 3 except that sufficient thiol to give the concentration indicated was added in a volume not exceeding 1.5 ml. and the volume of phosphate buffer adjusted to maintain a combined volume of 8.0 ml. The Table summarizes the results of four experiments in which different thiols were compared with mercaptoethanol and in which different concentrations of Factor B (between 5 and 9 μM) were used. Oxidation rates are expressed as in Fig. 4. 1 cm. $\equiv$ 0.48 $\mu\text{l.}$ of O_2 /min. (Expt. 1) or 0.43 $\mu\text{l.}$ of O_2 /min. (Expts. 2-4). 'Relative corrected rate' is the oxidation rate with Factor B, corrected by subtraction of the blank without Factor B, and expressed as a percentage of the corrected rate for mercaptoethanol in the same experiment.

Expt. no.	Thiol	Concn. (mm)	Oxidation rate (cm./min.)		Relative corrected rate
			Without Factor B	With Factor B	
1	2-Mercaptoethanol	30	0.10	3.55	100
2		30	0.10	6.60	
3		30	0.10	6.00	
4		30	0.15	5.75	
1	2,3-Dimercaptopropanol	30	2.20		
		10	1.05	2.95	52
1	Thioglycollic acid	150	0.40	2.55	62
		30	0.05	1.10	30
1	Cysteine	30	0.15	0.85	20
2	Glutathione	30	0.30	1.05	12
3	Dihydro-6-thioctic acid	5	0.20	10.8	180
		2.5	0.10	6.20	103
4	Dihydro-6-thioctic acid amide	2.5	0.20	9.7	170
		1.25	0.15	6.00	104
4	Ascorbic acid	30	0.20	0.20	0

Table 3. *Catalytic activity of vitamin B₁₂ derivatives in oxidation of 2-mercaptoethanol*

All samples were tested under the conditions of Fig. 3. For acid treatment, samples in 0.6 ml. of 1.9N-HCl were heated at 65° for 30 min., then added to 3.5 ml. of 70 mM-phosphate buffer, pH 7.1, containing NaOH equivalent to the HCl and cooled, and suitable portions tested. The catalyst concentrations tested were such as to give an oxidation rate of at least 3.0 cm./min. except with the untreated lactone, monobasic acids and dibasic acids where the rates were from 0.95 to 2.15 cm./min. The activities after acid treatment are calculated per $\mu\text{m-mole}$ of the original untreated derivative. Oxygen uptakes are uncorrected for the effect of the solutes on the solubility of oxygen. Fuller details of the structure of the coenzyme form are given by Lenhart & Hodgkin (1961) and of the other derivatives by Smith (1960).

Compound no.	Derivative	Structural relationship to cyanocobalamin	Activity ($\mu\text{l.}$ of O_2 uptake/min./ $\mu\text{m-mole}$ of catalyst)	
			Untreated	After acid treatment
(1)	Cyanocobalamin (vitamin B ₁₂)	—	0.081	129
(2)	Dimethylbenzimidazolylcobamide coenzyme	CN group replaced by 5'-deoxy-adenosine	0.027	60
(3)	Hydroxocobalamin	CN group replaced by -OH	0.79	65
(4)	Methylamide	One amide group of side chains methylated	3.4	107
(5)	Anilide	One amide group of side chains phenylated	4.0	100
(6)	Lactam	Acetamide side chain c converted into lactam ring fused to ring b	0.62	198
(7)	Lactone	Lactone corresponding to lactam	0.57	59
(8)	Monobasic acids	Mixture of derivatives with one NH_2 group lost from side chains	1.10	92
(9)	Dibasic acids	Mixture of derivatives with two NH_2 groups lost from side chains	0.94	34
(10)	Tribasic acids	Mixture of derivatives with three NH_2 groups lost from side chains	3.0	50
(11)	Factor A	5,6-Dimethylbenzimidazole replaced by 2-methyladenine	3.4*	169
(12)	Factor B	Nucleotide absent†	317	
(13)	Factor D	Structure unknown	200†	93

* Maximum rate (see text).

† Sample used also contained dicyanocobinamide (see text).

‡ Based on an assumed molecular weight of 1000.

Some uncertainty exists about the precise activities of these last two derivatives. The constitution of Factor D is unknown and the activity quoted is based on an assumed molecular weight of 1000. The Factor B preparation was a mixture of monocyano- and dicyano-cobinamide, and the activities of the individual forms were not determined. Previous manometric tests with a preparation containing only the monocyano form showed it to be highly active (Peel, 1962*a*). Dicyanocobinamide may be inactive (see below), but it is nevertheless evident that removal of the nucleotide moiety from the cyanocobalamin molecule gives an enormous increase in catalytic activity. In consequence, the possibility cannot be rigidly excluded that the activities observed with derivatives nos. 1-11 might be partly or wholly due to cobinamide impurities, as the highest activity (no. 5) would only require approx. 1% of such impurity. This possibility is regarded as unlikely with cyanocobalamin and hydroxocobalamin; these materials were crystalline, they were tested more extensively than the other derivatives and fresh solutions showed no significant increase in activity over several hours.

Effect of acid treatment. When cyanocobalamin is warmed briefly with strong acid, the nucleotide is lost and Factor B is one of the main products (Smith, 1960). Since Factor B has a much higher catalytic activity than cyanocobalamin, it was decided to see whether a simple acid treatment could be used to enhance the activity of other vitamin B₁₂ derivatives. When cyanocobalamin was heated with hydrochloric acid at 65°, maximum activity was reached after 20 min. It was only 40% of that expected from an equivalent amount of Factor B (Table 3), possibly owing to the formation of less active derivatives by side reactions. A standard treatment of 30 min. was adopted and in all cases except for Factor D a large increase in activity was observed (Table 3). The final activities were not identical but the range after acid treatment (approx. sixfold) is very much smaller than with the untreated derivatives. The activity of Factor D was halved by acid treatment and this, coupled with its original high activity, suggests that, like Factor B, it may contain no nucleotide.

Effect of cyanide. Since cyanocobalamin is much less active than hydroxocobalamin and since cyanide has been extensively used in extraction procedures for vitamin B₁₂, the effect of added cyanide on mercaptoethanol oxidation was examined. With 9 μ M-Factor B as catalyst, cyanide had very little effect at concentrations less than 1 μ M; at a concentration of 1 μ M there was a marked inhibition and this became complete at a concentration of 0.1 mM (Table 4). Complete inhibition at this concentration was also observed with cyanocobalamin (18 μ M). These inhibitions are most easily explained

as being due to the formation of inactive dicyano forms. A considerable excess of cyanide may be needed to stabilize such derivatives in the test system since one possible action of the mercaptoethanol is a rapid removal of cyanide groups from cobinamides. With cyanocobalamin, no removal of cyanide was detectable spectrophotometrically within several minutes of adding mercaptoethanol, but a slow conversion into the more active hydroxocobalamin could account for the increasing rate of catalysis with cyanocobalamin in the manometric experiments (Fig. 2).

Effect of metals, ethylenediaminetetra-acetate and yeast extract. In view of the ability of heavy-metal ions to catalyse the oxidation of thiols (e.g. Warburg, 1949), Co²⁺ ions and a number of other metal ions were tested in the present system both for catalytic activity and for possible interference with the reaction when Factor B is the catalyst: Mn²⁺, Cr³⁺, MoO₄²⁻ and Fe³⁺ ions at a concentration of 1 mM were all inert (Table 5); Co²⁺, Ni²⁺ and Cu²⁺ ions all showed catalytic activity, Co²⁺ ions being the most active, the initial rate with 10 μ M corresponding to an activity of 0.35 μ l. of oxygen/min./ μ mg. ion, which is considerably higher than that of cyanocobalamin. With this concentration of Co²⁺ ion, however, the rate fell off sharply as the reaction progressed (Fig. 3), and at the same time the reaction mixture turned yellow-brown. This fall in rate was due to loss of catalyst rather than to the fall in oxygen concentration. This became evident on re-oxygenating at the end of the reaction by slightly raising the plunger and increasing the stirring rate for a brief period. The subsequent oxidation rate was only about one-tenth of that when the Co²⁺ ion was first added. This rapid fall in activity explains why no catalysis was detectable with 10 μ M-Co²⁺ ion in manometric tests (Peel, 1962*a*). A marked falling off of the rate was also observed with 0.1 mM-Cu²⁺

Table 4. *Effect of cyanide on the oxidation of 2-mercaptoethanol*

Potassium cyanide to give the final concentration indicated was added in a volume of 0.08 ml. to the phosphate buffer. Other details were as for Fig. 3. The oxidation rate is expressed as in Fig. 4 and corrected by subtraction of blank rate in the absence of added catalyst (0.1 cm./min.). 1 cm. $\equiv$ 0.36 μ l. of O₂/ml.

Catalyst	Concn. of cyanide (M)	Corrected oxidation rate (cm./min.)
Factor B (9 μ M)	.	6.1
	10 ⁻⁸	6.5
	10 ⁻⁷	6.3
	10 ⁻⁶	4.2
	10 ⁻⁵	0.65
	10 ⁻⁴	0
Cyanocobalamin (18 μ M)	.	2.9
	10 ⁻⁴	0

Table 5. *Effect of metal ions, ethylenediaminetetra-acetate and yeast extract on the oxidation of 2-mercaptoethanol*

The metal ions were added as MnSO_4 , $\text{CrK}(\text{SO}_4)_2$, $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$, $\text{Fe}_2(\text{SO}_4)_3$, CuSO_4 , NiSO_4 and CoSO_4 respectively. In Expt. 1, metal ions and extract of boiled yeast were introduced in a volume of 0.08 ml. in the same manner as the vitamin B_{12} derivatives. EDTA was added with the phosphate buffer; likewise the yeast extracts in Expt. 2. When Factor B and metal ions or yeast extracts were both present, the Factor B (final concn. approx. $9 \mu\text{M}$) was added last in a volume of 0.04 ml. Concentrations (M) of metal ions in the Table and in the text are calculated as g.ions/l. Other details were as for Fig. 3; oxidation rates are expressed as in Fig. 4. 1 cm. $\equiv$ 0.48 (Expt. 1) or 0.45 (Expt. 2) $\mu\text{l.}$ of $\text{O}_2/\text{ml.}$

Expt. no.	Additions	Concn. (M)	1 mM-EDTA	Rate of oxidation (cm./min.)		
				No Factor B (a)	With Factor B (b)	(b - a)
1	None	.	-	0.1	6.1	6.0
		.	+	0.1	6.0	5.9
	Mn^{2+} ion	10^{-3}	-	0.1	6.1	6.0
	Cr^{3+} ion	10^{-3}	-	0.1	6.0	5.9
	MoO_4^{2-} ion	10^{-3}	-	0.1	5.7	5.6
	Fe^{3+} ion	10^{-3}	-	0.1	5.7	5.6
	Cu^{2+} ion	10^{-5}	-	0.5	6.1	5.6
			+	0.5	.	.
		10^{-4}	-	1.0*	.	.
			+	0.8	.	.
	Ni^{2+} ion	10^{-5}	-	0.5	8.0	7.5
			+	0.05	5.8	5.75
		10^{-4}	-	4.6	.	.
			+	0.1	.	.
	Co^{2+} ion	10^{-6}	-	0.55	6.4	5.85
		10^{-5}	-	7.2*	.	.
			+	0.1	.	.
	Extract of boiled yeast	0.3 mg./ml.	-	0.35	6.2	5.85
			+	0.1	.	.
2	None	—	-	0.1	6.5	6.4
	Extract of	6.3 mg./ml.	-	0.3	6.1	5.8
	boiled yeast	21 mg./ml.	-	0.55	5.5	4.95
			+	1.65	6.0	4.35
	Difco yeast	2.1 mg./ml.	-	0.2	1.2	1.0
	extract		+	0.1	1.2	1.1

* Initial rate; the rate fell progressively with time.

ion, but not with Ni^{2+} ion although a red-brown colour developed with the latter. At concentrations giving low catalytic rates ($10 \mu\text{M}$ for Cu^{2+} and Ni^{2+} ions; $1 \mu\text{M}$ for Co^{2+} ion), Cu^{2+} and Co^{2+} ions had very little influence on the rate due to Factor B [(b - a) in Table 5], but Ni^{2+} ion caused a significant enhancement.

EDTA (1 mM) completely reversed all the effects observed with Ni^{2+} and Co^{2+} ions but barely inhibited the catalysis with Cu^{2+} ions. It did not affect the reaction with Factor B. The effect of metal-ion-binding agents on the blank oxidation with mercaptoethanol in the absence of added catalyst was also investigated, 0.15M-mercaptoethanol being used so as to give a more easily measured rate. Little if any effect was observed with 10 mM-EDTA, 1 mM-sodium azide, 1 mM-1,10-phenanthroline or 0.1 mM-potassium cyanide.

To gain some indication whether other biological materials affect mercaptoethanol oxidation, a boiled extract of baker's yeast was used, as this organism

contains almost no vitamin B_{12} (Jukes & Williams, 1954). When tested in the same way as the metal ions, 0.3 mg./ml. gave a slight catalysis that was abolished by 1 mM-EDTA and did not interfere with the catalysis by Factor B (Table 5: Expt. 1). Higher concentrations were tested by adding freeze-dried material to the buffer. These gave little further increase in the blank rate (a); the chief effect was an inhibition of the rate due to Factor B which became marked at 21 mg./ml. (Table 5: Expt. 2). Difco yeast extract inhibited much more (80 % at 2.1 mg./ml.). EDTA caused an unexpected increase in the blank rate with the highest concentration of boiled extract, but did not relieve the inhibitions. The blank rate may be due, in part, to metalloporphyrin derivatives, since haematin catalyses the oxidation of cysteine (Harrison, 1924; Krebs, 1929). In preliminary experiments with the author's system, haematin catalysed mercaptoethanol oxidation with an activity similar to that of cyanocobalamin.

Spectral studies

When cyanocobalamin or hydroxocobalamin solutions are allowed to stand with thioglycollate at alkaline pH, the colour changes from pink to orange-brown, but on subsequent shaking the pink colour is instantly restored. This process can be repeated indefinitely, and Smith (1960) pointed out that under these conditions the cobalamin catalyses the atmospheric oxidation of the thioglycollate. The orange-brown derivative is almost certainly vitamin B_{12r}, a compound first produced by the reduction of cyanocobalamin with hydrogen (Diehl & Murie, 1952). Beaven & Johnson (1955) also state that a compound with the same spectrum is produced when cyanocobalamin is treated with cysteine at alkaline pH. The structure of vitamin B_{12r} is unknown but it is rapidly oxidized by air to give hydroxocobalamin and there is evidence that it contains bivalent cobalt as opposed to the trivalent cobalt in cyanocobalamin. The alternate formation and oxidation of vitamin B_{12r} could thus account for the catalysis of thioglycollate oxidation by cyanocobalamin and hydroxocobalamin. A similar mechanism appeared possible in mercapto-

ethanol oxidation and spectral evidence for a reduced catalytic intermediate was therefore sought. A marked change in the spectra of Factor B and of hydroxocobalamin after a few minutes with mercaptoethanol at neutral pH had been noted at an early stage in the investigation. Hydroxocobalamin was selected for more detailed study as it was more easily available, the material was crystalline and what little information is available on the reduction of vitamin B₁₂ derivatives refers to cobalamins. Cyanocobalamin was not used because of its low catalytic activity and because it does not give detectable spectral changes in a short time at neutral pH.

The situation at neutrality proved to be more complicated than was anticipated and a simple picture only emerged under alkaline conditions, where colour changes similar to those with thioglycollate were observed. The colour change from pink to orange-brown was accompanied by the disappearance of the absorption peak of hydroxocobalamin in the 350–360 m μ region. At pH 11 this maximum lies at 360 m μ and the progress of the reaction was followed by extinction measurements at this wavelength. The reaction was complete after 15 min. and the spectrum of the product (Fig. 6) was almost identical with that of vitamin B_{12r}, the wavelengths of maximum absorption being 313, 405 and 475 m μ , in close agreement with the values of 312.5, 405 and 473 given by Diehl & Murie (1952). On shaking with air, the original pink colour was temporarily restored and these colour changes could be repeated indefinitely. Vitamin B_{12r} thus appears to be a catalytic intermediate in the oxidation of mercaptoethanol under these conditions.

It was initially believed that similar processes occur at neutral pH (Peel, 1962*a*) on the basis of preliminary spectral data with hydroxocobalamin and Factor B. For technical reasons, observations at that time were limited to the region above 340 m μ , and more detailed experiments did not support the previous conclusions. First, a marked change in colour, to violet, was noted immediately on adding the mercaptoethanol. This was followed by a slow change to a yellow-brown over a period of several minutes and this colour remained unchanged on shaking with air, even when limiting amounts of thiol were used. In addition, a further slow change to yellow-green became apparent after about 1 hr. Secondly, when the spectrophotometric examination was extended to lower wavelengths it became clear that the yellow-brown product was not vitamin B_{12r}. The progress of the reaction was followed at 353 m μ , this being the maximum for the near ultraviolet peak of hydroxocobalamin at pH 7.1. After 20 min., the initial rapid fall in extinction had ceased but a slow drift was still detectable; scanning of the spectrum was begun at this point.

Fig. 6. Effect of 2-mercaptoethanol on the spectrum of hydroxocobalamin. Spectrophotometer cells of 1 cm. light-path contained initially, in 2.9 ml.: 100 μ moles of potassium phosphate buffer, pH 7.1, or 100 μ moles of Na₂CO₃ (giving a final pH of 11), and 0.1 μ mole of hydroxocobalamin; 0.1 ml. of 0.2M-mercaptoethanol was added at zero time. Measurements were made at room temperature (23–25°). Reference cells contained 33 mM-phosphate buffer or 33 mM-Na₂CO₃. Curves are continuous traces from the Optica recording spectrophotometer: —, at pH 7.1 before adding mercaptoethanol; ---, at pH 7.1, 20 min. after adding mercaptoethanol; ·····, at pH 11, 15 min. after adding mercaptoethanol. Times are those after which recording of the spectrum began; the time required to record each spectrum was approximately 6 min.

The large peak at 313 $m\mu$ characteristic of vitamin B₁₂ was absent, although there was a small shoulder at this wavelength. Neither did the spectrum show any resemblance to the reduced cobalamin derivative of Boos, Carr & Conn (1953) which is produced by treating cyanocobalamin with an excess of chromous derivatives and has absorption maxima at 385, 460 and 554 $m\mu$.

These experiments show that the action of mercaptoethanol on hydroxocobalamin at neutral pH is complex and is not limited to the formation and reoxidation of a reduced derivative such as vitamin B₁₂. The mechanism of catalysis at neutral pH thus remains obscure.

DISCUSSION

The quantitative findings presented above show that the catalytic effect of vitamin B₁₂ derivatives on thiol oxidations can be very potent and permit some assessment of the possible implications and applications of this activity. The magnitude of the reaction in a given situation is greatly influenced by such factors as pH and the nature of the thiol and vitamin B₁₂ derivative, but it is evident that significant effects may be expected under conditions likely to be encountered experimentally. This catalysis was observed with all the thiols so far tested; most of these are frequent components of incubation mixtures and some are of natural occurrence, including the thioctic acid derivatives which were by far the most readily oxidized thiols. Present indications are that the predominant natural forms of vitamin B₁₂ in living cells are the coenzymes and other cobamides containing a nucleotide (e.g. Volcani, Toohey & Barker, 1961; Toohey & Barker, 1961); in general, these compounds are relatively poor catalysts of thiol oxidation. The much more active cobinamides are, however, known to occur naturally, e.g. in faeces and in rumen contents (Ford & Porter, 1953), and they may also arise as metabolic intermediates or as impurities in cobamide preparations.

Three consequences of non-enzymic interactions between thiols and vitamin B₁₂ derivatives may affect the interpretation of experimental observations obtained *in vitro*. First, catalytic oxidation may lead to an unsuspected removal of reduced thiol. This might perhaps be of importance where small amounts of thiol are commonly used, e.g. with thioctic acid or with other thiols not so far tested such as CoA or proteins. Secondly, such reactions may result in a rapid removal of dissolved oxygen from solution, with accompanying effects on enzyme systems. Thus the requirement for vitamin B₁₂ derivatives in the CO₂-pyruvate exchange reaction observed with bacterial preparations in the presence of mercaptoethanol (Rabinowitz, 1960;

Rabinowitz & Allen, 1961) can be best explained as the protection of a labile enzyme by the removal of dissolved oxygen, rather than as a true coenzyme requirement (Peel, 1962*a*). Thirdly, the action of thiols, in catalytic oxidation processes or otherwise, may convert vitamin B₁₂ derivatives into products having different biological activities.

The physiological significance of thiol oxidations catalysed by vitamin B₁₂ derivatives is more difficult to assess. These reactions bear no strict parallel with any of the known enzymic reactions requiring vitamin B₁₂ coenzymes, and the one coenzyme tested in the non-enzymic thiol oxidation had very low activity. Catalytic mechanisms involving the oxidation and reduction of cobamides have been proposed for the succinyl-CoA-methylmalonyl-CoA interconversion (Eggerer, Stadtman, Overath & Lynen, 1960), the glycol-aldehyde interconversions (Brownstein & Abeles, 1961) and the methylation of homocysteine (Guest, Friedman, Woods & Smith, 1962), and it is possible that thiol groups might be involved in these oxido-reductions. The formation of disulphide linkages in proteins is a physiologically important thiol oxidation in which vitamin B₁₂ derivatives might play a part, either enzymically or non-enzymically. On the other hand, the available evidence with intact organisms suggests that vitamin B₁₂ promotes the reduction of thiols (Smith, 1960). For example, Dubnoff & Bartron (1956) found that the concentration of protein thiol groups in washed cells of the *Escherichia coli* mutant 113-3 fell after aging but was restored on incubating with vitamin B₁₂, especially in the presence of glutathione. A possible explanation of such effects is that vitamin B₁₂ derivatives may catalyse oxidation-reduction reactions between different thiols or the reduction of thiols by other hydrogen donors.

The susceptibility to oxygen of the CO₂-pyruvate exchange system from *P. elsdennii* appears similar to that reported for cytochrome *c* reductase (Dixon, Maynard & Morrow, 1960) and yeast lactate dehydrogenase (Armstrong, Coates & Morton, 1960), in that inactivation is rapid and irreversible (Peel, 1962*a*; and unpublished work). The effectiveness of mercaptoethanol together with vitamin B₁₂ derivatives in maintaining the activity of the CO₂-pyruvate exchange system suggests that this combination of reagents may prove of wider use in handling other enzymes showing this type of sensitivity to oxygen.

A further use of these reagents as 'oxygen scavengers' is in the culture and handling of strictly anaerobic micro-organisms. The addition of mercaptoethanol (4 mM) and Factor B (1 μ M) to culture media a few minutes before inoculation has already been successfully used to establish and maintain anaerobiosis in nutritional studies with

P. elsdensis involving growth from small inocula (H. J. Somerville and J. L. Peel, unpublished work). This method of removing oxygen has the advantage that the thiol remains relatively stable until the vitamin B₁₂ derivative is added but that thereafter oxygen is removed very rapidly.

The polarographic method permits a convenient and reasonably precise measurement of auto-oxidation rates, and the kinetics of mercaptoethanol oxidation are suitable for a quantitative determination of vitamin B₁₂ derivatives. A determination based on this principle would be highly sensitive, at least with cobinamides. Thus a rate of about 1 cm./min. is given by 10 μ mg. of Factor B, and with a smaller reaction chamber the sensitivity could be brought close to that of microbiological assays where 0.1–0.5 μ mg. is a common range. The sensitivity with cobalamins and other common cobamides would be less but could be increased considerably by prior treatment with acid. The individual vitamin B₁₂ derivatives have different activities in thiol oxidation, even after acid treatment, but the same is true of other assays and differences in activity are in themselves useful in studying interconversions. The mercaptoethanol-oxidation method would have an important advantage in speed over microbiological assays and would be applicable to a wider range of vitamin B₁₂ derivatives. For instance, it could be used with Factor D, which is devoid of biological activity apart from antagonism in the chick assay (Smith, 1960). On present evidence, the most serious interfering substances are likely to be cyanide and an unidentified inhibitor present in yeast extracts. The effects of acid treatment, cyanide and EDTA are properties that might be useful in distinguishing catalysis by vitamin B₁₂ derivatives from spurious reactions. Where the proportion of interfering substances is low, a method of determination of vitamin B₁₂ derivatives would thus appear both feasible and a useful addition to existing assay procedures.

SUMMARY

1. At neutral pH, vitamin B₁₂ derivatives catalyse the auto-oxidation of 2-mercaptoethanol to the disulphide form. The effect can be readily demonstrated manometrically but this method does not give reliable rate measurements because of side reactions.

2. A polarographic apparatus suitable for measuring changes in the partial pressure of oxygen of a few minutes' duration is described and has been used to study mercaptoethanol oxidation.

3. At pH 7.1 and with all vitamin B₁₂ derivatives tested except Factor A, the fall in oxygen concentration is linear with time for the greater part of the reaction. The final oxygen pressure reached is less than 2 mm. Hg.

4. With Factor B as catalyst, the oxidation rate increases with pH from nearly zero at pH 6.0 to a maximum at pH 8.0, and increases approximately fourfold between 25° and 37°. The rate shows a Michaelis-type dependence on mercaptoethanol concentration (half-maximum velocity at about 30 mM) and is directly proportional to catalyst concentration.

5. At 37° and pH 7.1 with Factor B as catalyst, activity with different thiols increased in the order: glutathione; cysteine; thioglycollate; 2-mercaptoethanol; 2,3-dimercaptopropanol; dihydro-6-thioctic acid; dihydro-6-thioctic acid amide.

6. With mercaptoethanol, the approximate molar catalytic activities of different vitamin B₁₂ derivatives relative to cyanocobalamin (1.0) are: dimethylbenzimidazolylcobamide coenzyme, 0.3; hydroxocobalamin, 10; sundry cobalamin analogues, 7–50; Factor B, 4000; Factor D, 2500. Activities of all the cobamide derivatives are greatly enhanced by brief heating with acid.

7. Co²⁺, Ni²⁺ and Cu²⁺ ions catalyse the oxidation of mercaptoethanol; yeast extract has slight catalytic activity. EDTA (1 mM) completely abolishes the effect of Co²⁺ or Ni²⁺ ions, but not that of Cu²⁺ ion. Catalysis by Factor B is unaffected by 1 mM-EDTA, but is completely inhibited by 0.1 mM-potassium cyanide. Large concentrations of yeast extract interfere with the reaction.

8. Spectral studies with hydroxocobalamin and mercaptoethanol indicate that vitamin B₁₂ is a catalytic intermediate in thiol oxidation at pH 11. At neutrality the situation is obscured by side reactions.

9. The implications of these reactions *in vitro* and *in vivo* and their possible use for establishing anaerobic conditions and in the determination of vitamin B₁₂ derivatives are discussed.

I am grateful for the following help: gifts of materials from the donors specified in the text, advice on chemical matters (Dr E. Lester Smith and Dr J. W. G. Porter) and on polarography (Dr J. D. Jones), technical assistance (Mrs C. Drabble and Mr R. Bacon), and partial support of the work (Rockefeller Foundation).

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The Metabolism of Progesterone in Man

EXTRACTION AND SEPARATION BY COUNTERCURRENT DISTRIBUTION OF THE METABOLITES IN URINE

By R. A. HARKNESS AND K. FOTHERBY*

Clinical Endocrinology Research Unit (Medical Research Council), University of Edinburgh

(Received 11 February 1963)

After the administration of [4-¹⁴C]progesterone or [16-³H]progesterone to humans, 15–61 % of the dose was recovered from the urine and 13–28 % from the faeces (Gallagher *et al.* 1954; Pearlman, 1957; Davis & Plotz, 1958; Sandberg & Slaunwhite, 1958). The known metabolites of progesterone in urine, 5 β -pregnane-3 α ,20 α -diol (pregnanediol), 3 α -hydroxy-5 β -pregnan-20-one (pregnanolone) and their isomers, account for less than one-third of the administered hormone, so that much of the radioactivity present in urine must reside in compounds other than the above metabolites, if hydrolysis of the conjugates of the above steroids in urine has been complete. In an attempt to identify these unknown metabolites the metabolism of [4-¹⁴C]-progesterone in man has been investigated. The present paper describes a study of the hydrolysis and extraction of the metabolites of [4-¹⁴C]-progesterone from the urine of ovariectomized

adrenalectomized women who had received the labelled hormone, and the preliminary separation of these metabolites by countercurrent distribution.

METHODS

Subjects

The subjects were five women (aged 41–59 years) who had been adrenalectomized and ovariectomized for mammary carcinoma. All subjects were in hospital during the investigation and were ambulant; they were receiving 25–37.5 mg. of cortisone acetate daily, by mouth, as replacement therapy. There was no evidence of impairment of hepatic or renal function. All subjects collected complete 24 hr. samples of urine for 9–14 days from the day of injection of [4-¹⁴C]progesterone.

Administration of [4-¹⁴C]progesterone

Subject A.W. received 20 μ C (0.3 mg.) of [4-¹⁴C]progesterone (The Radiochemical Centre, Amersham, Bucks.) intravenously over about 15 min. The steroid was administered by dissolving it in 20 ml. of 0.9 % sodium chloride in

* Present address: Postgraduate Medical School, Ducane Road, London, W. 12.