reaction in cancellous as compared with cortical bones, where the reaction is restricted to a few surface layers, but it does not explain the difference in the rate of renewal between the cancellous bones of the rabbit and cat. There must be other factors, apart from structural ones, on which the rate of mineral salt turnover depends.

Bone-salt renewal occurs in mature shaft bones mainly on the marrow side, i.e. in the same area where resorption is known to take place. Whether the two processes are connected with each other, e.g. through strain and stress, cannot be decided from the present experiments. But the close vicinity of such opposite processes rather suggests a cellular regulation (Newton, 1939).

SUMMARY

1. The rate of tracer uptake in long bones varies according to species; the incorporation of ^{32}P is considerably slower in the cat than in the rabbit or rat.

2. No decrease of tracer concentration occurs in the cortical parts of the long bones of the rabbit, cat or rat, or in the cancellous parts of the two former species in the course of 30 days. The decrease of the specific activity of phosphorus in the cancellous bones of the rat is explained by the continued skeletal growth of this animal in adult age.

3. The species differences are much better demonstrated with a newly developed technique, which affords an almost complete separation of the newly labelled bone salts from old-established bone. Large differences were found in the function of various parts of the shaft.

4. The different aspects of bone renewal in the adult animal, such as initial uptake, recrystallization,

Haversian replacement, mobilization of fixed isotopes, equilibrium between bone and blood minerals, are discussed in the light of the new observations.

I take great pleasure in thanking Dr H. J. Rogers for his continued interest in this work and for many helpful suggestions. Thanks are also due to Mr R. W. Lofthouse and Miss E. B. Morley for excellent technical assistance.

REFERENCES

- Amprino, R. (1952). *Experientia*, **8**, 20, 380.
 Amprino, R. & Engström, A. (1952). *Acta anat.* **15**, 1.
 Bauer, G. C. H. (1954). *J. Bone Jt. Surg.* **36A**, 375.
 Engfeldt, B., Engström, A. & Zetterström, R. (1952). *Biochim. biophys. Acta*, **8**, 375.
 Hevesy, G., Levy, H. B. & Rebbe, D. H. (1940). *Biochem. J.* **34**, 532.
 Leblond, C. P., Wilkinson, G. W., Bélanger, L. F. & Robichon, J. (1950). *Amer. J. Anat.* **86**, 289.
 Martland, M. & Robison, R. (1926). *Biochem. J.* **20**, 847.
 Neuman, W. F. & Mulryan, B. J. (1952). *J. biol. Chem.* **185**, 705.
 Neuman, W. F. & Neuman, M. W. (1953). *Chem. Rev.* **53**, 1.
 Neuman, W. F. & Riley, R. F. (1947). *J. biol. Chem.* **168**, 545.
 Neuman, W. F. & Weikel, J. H. (1954). *Atomic Energy Report*, UR-314.
 Newton, W. H. (1939). *Recent Adv. Physiol.* 6th ed. London: Churchill.
 Rogers, H. J. & Weidmann, S. M. (1951). *Brit. dent. J.* **90**, 7.
 Rogers, H. J., Weidmann, S. M. & Jones, H. G. (1953). *Biochem. J.* **54**, 37.
 Singer, L., Armstrong, W. D. & Premer, M. L. (1952). *Proc. Soc. exp. Biol., N.Y.*, **80**, 643.
 Weidmann, S. M. & Rogers, H. J. (1950). *Biochem. J.* **47**, 493.
 Zetterström, R. (1952). *Biochim. biophys. Acta*, **8**, 283.

Determination of Cocarboxylase in Blood

BY W. W. KAY AND K. C. MURFITT

The Mental Hospitals' Group Laboratory, West Park Hospital, Epsom, Surrey

(Received 25 July 1955)

Cocarboxylase estimation has received considerable attention from Ochoa & Peters (1938) and Westenbrink & Steyne-Parvé (1949), among others. Most of the accepted methods employ alkali-washed yeast apoenzymes activated by excess of thiamine, and some also include iodoacetate to prevent the phosphorylation of added thiamine (Blanchaer & Cohen, 1949). Goodhart & Sinclair (1939) developed a method for estimating cocarboxylase in heated blood, and Westenbrink & Steyne-Parvé

(1949) used acid to extract cocarboxylase from blood, before applying their 'two-stage' method for assay.

In a system such as heated blood, which contains diphosphopyridine nucleotide and adenosine triphosphate in sufficient quantity to allow cocarboxylase synthesis by yeast enzymes (Lipton & Elvehjem, 1939), it seems unwise to include excess of thiamine. Further, now that carboxylase is recognized as a sulphydryl enzyme, which is

inhibited by iodoacetate (Stoppani, Actis, Deferrari & Gonzalez, 1953), it seems reasonable to exclude iodoacetate also from cocarboxylase-assay systems.

We have found alkali-washed yeasts to be unreliable sources of apoenzymes; with substances contained in blood they gave side reactions that could result in spurious evolution of CO_2 . It was therefore decided to apply a purified apocarboxylase preparation (Green, Herbert & Subrahmanyam, 1941) in the study of cocarboxylase estimation, in the hope that a better assay system could be produced.

The method to be described here introduces the use of Norite charcoal to eliminate an enzyme blank by adsorbing the cocarboxylase from alkaline solution during enzyme preparation, and the use of cyanide or thiosulphate to overcome the effects of interfering substances, probably trace metals. An internal standard ensures identical conditions of activation in tests and standards, as both contain heated blood.

METHOD

Principle

Cocarboxylase in heated blood cells is recombined with apocarboxylase prepared from yeast, and the resulting holoenzyme is employed in the decarboxylation of pyruvic acid, by a manometric technique for the measurement of evolved CO_2 .

Reagents

Stock cocarboxylase standard, 100 $\mu\text{g./ml.}$ This can be kept at 4° for up to 4 weeks.

Dilute standard cocarboxylase. This is freshly prepared from stock so as to contain 0.1 $\mu\text{g./ml.}$ of phosphate buffer (pH 6.2).

0.1M Phosphate buffer, pH 6.2. This is made by mixing 203.4 ml. of 0.1M- KH_2PO_4 solution with 46.6 ml. of 0.1M- Na_2HPO_4 solution.

0.33M Sodium pyruvate. 2.0 ml. of pyruvic acid (purest commercial) is neutralized in water to pH 6.2 by adding about 2.5 ml. of 50% (w/v) NaOH and the whole is then diluted to 100 ml. with phosphate buffer, pH 6.2.

0.2M Sodium cyanide. 1% (w/v) NaCN in phosphate buffer is adjusted to pH 6.2 with H_3PO_4 .

Ionic-activating solution. 1 vol. of 0.1M- MnSO_4 solution is mixed with 9 vol. of 0.1M- MgCl_2 solution.

M Calcium acetate solution.

Apocarboxylase solution. This is prepared at the time of the experiments. Stock enzyme suspension (10 ml., prepared as described below) is centrifuged for about 0.5 hr. To the precipitate are added 1 ml. of ionic-activating solution, 1 ml. of 0.2M-NaCN solution and 8.5 ml. of phosphate buffer, pH 6.2. A solution of activated apocarboxylase, sufficient for ten Warburg vessels, is then obtained.

Stock enzyme. (a) *Stock yeast*. About 0.5 lb. of ordinary baker's yeast is washed with 5 vol. of tap water, filtered on a large Büchner funnel (Whatman no. 541 or 542 paper), broken up and dried thoroughly in a vacuum desiccator over conc. H_2SO_4 . This yeast keeps almost indefinitely. Brownish, hard yeasts usually give the best enzyme preparations.

(b) *Suspension*. Dried yeast (20 g.), finely powdered, is stirred into 180 ml. of 0.1M- Na_2HPO_4 (pH 8.5) at 37° . The smooth suspension formed is incubated at 37° for 2 hr. so that the carboxylase is released into the solution.

The yeast cells are centrifuged and washed once with twice their volume of phosphate solution (pH 8.5) before being finally rejected. The combined supernatants are adjusted to pH 9.0 with conc. NH_3 soln. (sp.gr. 0.880) and 4 ml. of M calcium acetate are stirred in to remove turbidity (Green *et al.* 1941). The calcium phosphate precipitate is removed by centrifuging for 2 min. To the cleared solution solid $(\text{NH}_4)_2\text{SO}_4$ (38 g./100 ml. of solution) is added to precipitate the enzyme, which is separated in the centrifuge at high speed for 1 hr.

The precipitated protein is then dissolved in 50 ml. of 0.1M- Na_2HPO_4 solution and, after adjustment to pH 9.0 with ammonia, 700 mg. of Norite charcoal is added and the mixture is stirred for about 10 min. to remove cocarboxylase dissociated from the enzyme. The charcoal is removed by centrifuging for 2 min., and the solution is treated with 2.0 ml. of M calcium acetate, as before, to clear it. Any floating material is removed by filtering the solution through a rapid paper, and $(\text{NH}_4)_2\text{SO}_4$ (38 g./100 ml. of solution) is again used to precipitate the enzyme. The resultant suspension, which should be adjusted to pH 6.2 with dilute HCl, is almost white and gives an enzyme of negligible blank. This suspension keeps for about 4 weeks at 4° . If the precipitated enzyme is separated, dissolved in phosphate buffer (pH 6.2) and freeze-dried, it keeps almost indefinitely (Murfitt, 1954).

Treatment of blood

Well-mixed heparinized blood (4 ml.) is transferred to a tube and centrifuged. After the volume has been marked, the plasma is rejected and accurately replaced by phosphate buffer, pH 6.2. The cells are resuspended in the solution by stirring with a glass rod, and, after the tube is stoppered firmly, it is heated for 3 min. in a boiling-water bath. When cool, 4 ml. of buffer is added and a smooth suspension obtained by stirring with a glass rod. Since plasma contains no cocarboxylase, 1 ml. of heated blood suspension is equivalent to 0.5 ml. of original whole blood.

Procedure

The assay procedure is conducted in duplicate in Warburg vessels to which the reactants are added in order, as indicated in Table 1.

For apoenzymes prepared with Norite charcoal the blank flasks are generally unnecessary.

After the Warburg manometers have been assembled they are equilibrated in the bath at 30° for 15 min. to allow time for holoenzyme formation. Afterwards pyruvate is tipped in from the side bulb at zero time, the taps are closed at 5 min., and readings are taken at 10 and 12 min. and then again at 40 and 42 min. This gives readings during steady evolution of CO_2 for two overlapping 30 min. periods, from which mean values can be obtained.

Calculation

Evolution is measured in $\mu\text{l.}$ of CO_2 /30 min.

If T represents the test evolution, B the blank evolution and S the internal-standard evolution of CO_2 ,

$$\text{Blood cocarboxylase} = \frac{T - B}{S - T} \times 10 \mu\text{g./100 ml.}$$

Table 1. *Blood-coccarboxylase assay system*

Warburg flasks are set up in duplicate with the materials listed, and CO_2 evolution at 30° is measured for 30 min. after addition of the pyruvate to the enzyme-blood-buffer mixture.

	Blank (ml.)	Test (ml.)	Internal standard (ml.)
Dilute coccarboxylase (0.05 $\mu\text{g.}$)	—	—	0.5
Phosphate buffer, pH 6.2	1.5	0.5	—
0.33M Pyruvate (side bulb)	0.5	0.5	0.5
Heated blood suspension	—	1.0	1.0
Activated enzyme solution	1.0	1.0	1.0

Table 2. *Effect of various additions upon coccarboxylase activity*

The enzyme control flask contained apococcarboxylase and pyruvate. Other flasks contained 1 $\mu\text{g.}$ of coccarboxylase in addition. The final volume in all flasks was made up to 3 ml. with phosphate buffer.

System	Additions (0.1 ml. of 0.1 M)	Evolution of CO_2 ($\mu\text{l.}/30 \text{ min.}$)
Enzyme control	—	24
Control + coccarboxylase	—	24
Control + coccarboxylase	Hexose diphosphate	25
Control + coccarboxylase	Glycine	28
Control + coccarboxylase	Citrate	37
Control + coccarboxylase	NaCN	41

RESULTS

Over the usual range of working conditions and up to 0.2 $\mu\text{g.}$ of coccarboxylase, CO_2 evolution bears a linear relationship to quantity of coccarboxylase (Fig. 1).

Fig. 1. Relationship between CO_2 evolution and quantity of coccarboxylase added to heated blood in a system containing cyanide and enzyme. The evolution at 0.0 $\mu\text{g.}$ is that due to the blood and the enzyme blank.

Coccarboxylase activity is generally lower in re-constituted holoenzyme than in naturally occurring holoenzyme, where it has been found experimentally to be 44 $\mu\text{l.}$ of $\text{CO}_2/\text{min.}/\mu\text{g.}$ of coccarboxylase at 30° (Green *et al.* 1941). We have found that the internal-standard activity can reach this figure when good enzymes are available, but generally the activity is about two-thirds of this value.

In the early stages of the work it was found that a basic system of apoenzyme and coccarboxylase showed very little activity. However, when heated blood was substituted for the synthetic coccarboxylase its coenzyme caused some evolution of CO_2 , and it was concluded that the apoenzyme was active when certain blood constituents were present. Table 2 shows the effects of various substances (some of which may be present in blood) upon the basic enzyme system without blood. Cyanide had the greatest effect upon the activity of the system and was therefore included in subsequent work. Thiosulphate shows an almost equal effect, but no summation results from a combination of these activators. Melnick & Stern (1940) have correlated such effects with the presence of copper in the synthetic coccarboxylase preparation.

When the activity of standard coccarboxylase with cyanide is compared with that of the same standard in the presence of blood and cyanide, the latter is still much higher, demonstrating that some of the blood activators may differ from cyanide in their action (Table 3). To ensure equal activation in tests and standards the internal standard was therefore adopted.

Attempts to produce an ideal external standard by additions of thiamine, citrate and cysteine to the system have failed, because the activity of coccarboxylase, though increased, was still less than that of the internal standard. Plasma, diluted with an equal volume of phosphate buffer, pH 6.2, and added to the 0.05 $\mu\text{g.}$ of coccarboxylase in place of heated blood cells, produced the best approximation to an external standard, since the activity

value was then very close to that of the internal standard used with the corresponding heated blood cells. It seems likely, however, that an external standard based on plasma might vary according to the pathological condition of the subject.

Plasma contains no cocarboxylase, but there is a small amount of thiamine which may exert an activating effect on cocarboxylase. A similar amount of thiamine is also present in the heated cells, and this may cause synthesis or activation of cocarboxylase. Thus what is actually measured in this method is the sum of cocarboxylase and

addition of iodoacetate would cause complications through competition by iodoacetate for the various sulphhydryl groups available. Thus when blood proteins are present iodoacetate inhibition of cocarboxylase is not always shown, although the phenomenon is quite definite in the simple enzyme system (Stoppani *et al.* 1953).

Another question that arose in the course of this work (cf. Murfitt, 1954) concerned the possibility of a direct reaction between iodoacetate and thiamine, which has been shown to exist in a sulphhydryl form (Zima & Williams, 1940). The isolation by Myrbäck, Vallin & Magnell (1945) of

Table 3. *Comparison of the effect of cocarboxylase, in the presence and absence of blood*

Cocarboxylase (1 $\mu\text{g.}$) was used as (a) an external standard, i.e. without blood; (b) an internal standard with heated blood. In both (a) and (b) the enzyme control consisted of apocarboxylase, pyruvate and cyanide. In all flasks the final volume was adjusted to 3 ml. with phosphate buffer.

System	Evolution of CO_2 ($\mu\text{l./30 min.}$)	
	Total	Extra due to 1 $\mu\text{g.}$ of cocarboxylase
(a) Enzyme control	16	—
Control + cocarboxylase (1 $\mu\text{g.}$)	19	3
(b) Enzyme control + heated blood	45	—
Control + heated blood + cocarboxylase (1 $\mu\text{g.}$)	85	40

thiamine, but this is still, usually, an indication of the nutritional state of the subject. In the blood of people not receiving therapeutic thiamine, the effect of the free thiamine in the cells is very small. Where large doses of thiamine are given, it is probable that cocarboxylase values are anomalously high because of synthesis from or activation by the free thiamine of the blood. Differential estimation of thiamine and cocarboxylase was attempted by using iodoacetate, but the results were not conclusive.

Blood cocarboxylase levels in human subjects. Our results from 105 patients of both sexes who showed no clinical evidence of thiamine deficiency give a normal range of 5–12 $\mu\text{g./100 ml.}$ of blood, with a mean of $7.0 \pm 1.7 \mu\text{g./100 ml.}$ Patients with clinical signs of thiamine deficiency gave values below this range before, and within the range after, thiamine administration. Goodhart & Sinclair (1939) gave the normal range as 7.0 ± 2.1 , and Westenbrink & Steyne-Parvé (1949), by their blood-extraction method, gave a range higher than this ($11.2 \pm 1.5 \mu\text{g./100 ml.}$). Other normal ranges are given by Smits & Florijn (1949).

DISCUSSION

Activation of cocarboxylase by heated blood cells or plasma is probably due partly to the sulphhydryl compounds present. Considering also the sulphhydryl nature of carboxylase, it is clear that the

the disulphide form of thiamine by oxidation with H_2O_2 at pH 7.5 suggests that this form may exist in significant quantity at physiological pH values. It therefore seems possible that iodoacetate and thiamine could react directly and that this could be a contributory explanation of the opposite effects of these substances in the carboxylase system.

The sulphhydryl form of cocarboxylase could supply the site of reaction between pyruvate and holocarboxylase, where there is a sulphhydryl group which may be reversibly blocked by iodoacetate and protected by pyruvate (Stoppani *et al.* 1953). At present, the intermediate steps in decarboxylation of α -keto acids are little understood, but in all cases the specific configuration of cocarboxylase is required (Ochoa, 1951). Speculation about the place of a thiazole-thiol equilibrium for cocarboxylase in decarboxylation, and the question as to whether or not this furnishes the sole, vital sulphhydryl group of holocarboxylase, is of interest. Comparison of such a system for simple decarboxylation with the lipothiamide oxidative decarboxylation system, present in some organisms (Reed, 1953), may help the understanding of this problem.

SUMMARY

1. A method for the estimation of blood cocarboxylase is described.

2. Innovations include the use of Norite charcoal to obtain a blank-free apoenzyme, the addition of

cyanide to the system, and the use of an internal standard during assay.

3. Plasma showed an activating effect on cocarboxylase.

4. Reference is made to the effects of thiamine and iodoacetate in the system.

5. The possible significance of the sulphhydryl form of cocarboxylase in decarboxylation is discussed.

We are grateful to Roche Products Ltd. for a timely gift of pure cocarboxylase.

REFERENCES

- Blanchaer, M. C. & Cohen, L. H. (1949). *Canad. J. Res. E*, **27**, 275.
- Goodhart, R. & Sinclair, H. M. (1939). *Biochem. J.* **33**, 1099.
- Green, D. E., Herbert, D. & Subrahmanyam, V. (1941). *J. biol. Chem.* **138**, 327.
- Lipton, M. A. & Elvehjem, C. A. (1939). *Cold Spr. Harb. Symp. quant. Biol.* **7**, 184.
- Melnick, J. L. & Stern, K. G. (1940). *Enzymologia*, **8**, 129.
- Murfitt, K. C. (1954). Thesis and Dissertation: University of London.
- Myrbäck, K., Vallin, I. & Magnell, I. (1945). *Svensk kem. Tidskr.* **57**, 124. [(1946) *Chem. Abs.* **40**, 4094.]
- Ochoa, S. (1951). *Physiol. Rev.* **31**, 56.
- Ochoa, S. & Peters, R. A. (1938). *Biochem. J.* **32**, 1501.
- Reed, L. J. (1953). *Physiol. Rev.* **33**, 544.
- Smits, G. & Florijn, E. (1949). *Biochim. biophys. Acta*, **3**, 44.
- Stoppani, A. O. M., Actis, A. S., Deferrari, J. O. & Gonzalez, E. L. (1953). *Biochem. J.* **54**, 378.
- Westenbrink, H. G. K. & Steyne-Parvé, E. P. (1949). *Int. Z. Vitaminforsch.* **21**, 461.
- Zima, O. & Williams, R. R. (1940). *Ber. dtsh. chem. Ges.* **73**, 941.

The Effect of pH on the Toxicity of Fluoroacetic Acid

By J. G. ALDOUS AND K. R. ROZEE

Department of Pharmacology, Dalhousie University, Halifax, N.S.

(Received 9 September 1955)

The mechanism of the toxic action of fluoroacetate provides one of the few examples of what has been called 'lethal synthesis' (Peters, 1952). Fluoroacetate is not toxic to enzymes (Bartlett & Barron, 1947), but its incorporation into the reactions of the Krebs cycle in place of acetate results in the production of a fluorinated tricarboxylic acid (in all probability, fluorocitric acid) which blocks the action of the enzyme aconitase (Peters, 1952). Since the reactions of the Krebs cycle are associated with the mitochondria of the cell (Nossal, 1953), the action of fluoroacetate must be an intracellular one.

For a number of years a project has been under way in this Laboratory concerning the interactions of the yeast cell and its environment, particularly where changes in pH are involved. The programme is based upon the premise that if the intracellular action of an ionizable chemical agent is found to vary with extracellular pH, then something can be learned about the internal environment of the cell. Iodoacetic acid was first applied to this problem and here pH was found not only to influence the degree of toxicity to yeast cells, but also to determine the characteristics of the inhibitory action observed (Aldous, 1948). The disadvantage of using iodoacetic acid was that its *in vivo* action was characterized by the inactivation of a variety of enzymes (Aldous, 1952). Fluoro-

acetic acid (HFA), on the other hand, possesses some pharmacological properties which would appear to make it a more suitable agent. This possibility, together with the object of establishing the effect of pH upon the toxicity of HFA to yeast cells, were the reasons for undertaking the present investigation. Yeast cells have been used as the biological material because they will tolerate wide fluctuations in environmental pH without showing appreciable changes in their respiratory metabolism (Barron, Ardao & Hearon, 1950).

A search of the literature showed that whereas a few investigators have noted an effect of pH upon the toxicity of fluoroacetate, none, with the possible exception of Black & Hutchens (1948), has made a systematic study of the phenomenon. These authors noted that as the pH of the medium was lowered from 6.0 to 4.0, the fluoroacetate-induced inhibition of the oxygen consumption of yeast cells was progressively increased. This action is attributed to the fact that for 'fluoroacetic acid (pK_a 3.81) a change in pH from 6.0 to 4.0 increases the concentration of undissociated fluoroacetic acid molecules 60-fold'. No reference for the pK_a is given, but from this value a K_a of 1.55×10^{-4} can be calculated. Chenoweth (1949) quotes a value of 21×10^{-4} for the K_a , which is more in keeping with those for the other halogen-acetic acids, and which yields a pK_a of 2.68. By using Chenoweth's figure,