The Characterization and Assay of Enzymes in Rat Adrenal Cortex

1. ESTERASE AND PHOSPHATASE ACTIVITIES

By J. J. GORDON

*Biochemical and Endocrinological Research Department, Bristol Mental Hospitals,
Fishponds, Bristol*

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There is evidence that significant changes may take place in the activities of enzymes present in secreting glands under the influence of regulatory hormones. Gutman & Gutman (1938) reported increases in the acid phosphatase activity of prostate during the development of the epithelia induced by testosterone. According to Fuenzalida (1949), prolonged oestrogen treatment causes an increase in alkaline phosphatase activity in guinea pig uterus. Large increases in arginase and alkaline phosphatase activities in the mammary gland during lactation have been found by Folley & Greenbaum (1947).

Evidence regarding the nature of similar changes taking place in the adrenal cortex has been largely histological. Characteristic alterations in lipid metabolism in this gland in the rat, following hypophysectomy, were indicated by the work of Reiss, Balint, Oestreicher & Aronson (1936). Variations in alkaline phosphatase content under different endocrine conditions have been investigated in the mouse and rat by Elftman (1947) and by Dempsey, Greep & Deane (1949). Chemical studies, however, have so far been limited, though isolated observations, sometimes of a qualitative kind, have from time to time been reported. The presence of a lipase (i.e. tributyrinase) in the tissue was demonstrated by Cheboksarov & Malkin (1927) and by Scoz & Mariani (1939); this would seem to be of importance in relation to the lipid changes observed by Reiss *et al.* (1936). Chemical proof of the existence of an adrenal cortical phosphomonoesterase has also been furnished (Granger & Bessoles, 1945).

A knowledge of the properties of the various enzyme systems present in the adrenal cortex, and the manner in which they are influenced by different endocrine factors, may be important in connexion with the processes governing the secretory activity of the gland. Investigations on these lines have accordingly been initiated, and the present paper contains an account of the experimental work so far carried out to characterize the lipase and phosphatase activities shown by aqueous homogenates of adrenal cortical tissue, and to devise suitable methods for their accurate assay in small quantities of tissue. Since tributyrin has been used as the principal test substrate for lipase, the latter will be

referred to throughout as the 'aliphatic esterase'. Some related observations on cholinesterase activity are included.

METHODS

Preparation of tissue homogenates

The demedullated adrenals of albino rats have been used exclusively as a source of enzyme. The rats were killed by decapitation, the carcasses exsanguinated, and the adrenals rapidly removed, cleaned from adherent fat, then opened laterally with fine scissors, and the medullae removed from both halves. The pooled demedullated adrenal tissue obtained from a number of rats was weighed, rapidly washed with cold water by decantation to remove excess blood, and homogenized in cold distilled water in a Potter-Elvehjem (1936) homogenizer. After making to the appropriate volume with distilled water and mixing thoroughly, the homogenate was allowed to stand 1-2 min. to allow the capsular debris to settle, then decanted carefully off.

Aliphatic esterase and cholinesterase activity

These were usually followed at 37° by use of the Ammon (1933) technique. Tissue homogenate (1 ml.) containing a suitable wet weight of tissue (12-18 mg. for aliphatic esterase and 40 mg. for cholinesterase) was incubated in NaHCO₃ (0.027 M) medium with the ester, the total fluid volume being 3 ml., in an atmosphere of 5% CO₂/N₂. CO₂ output was measured at regular intervals for a period of 30-60 min. and corrections made in each case for spontaneous hydrolysis. Activities are expressed as μ l. CO₂/30 min./g. In preliminary experiments, aliphatic esterase activity was tested against a series of simple esters; most of the experiments with this system, however, were performed with tributyrin as substrate. 0.2 ml. of the pure ester (more than sufficient to saturate the system) was used. For cholinesterase activity, the substrate consisted of acetylcholine (ACh, 6-12 mM), acetyl- β -methylcholine (MeCh, 12 mM) or benzoylcholine (BCh, 6 mM). It was confirmed that the ester concentrations were in every case adequate to ensure maximal hydrolysis rates. In some experiments selected inhibitors were included in the incubation mixture.

For following the hydrolysis of the glyceryl acetates, a variation of the titration method of Knaff-Lenz (1923) was employed at room temperature, on account of the rather high rates of spontaneous hydrolysis shown by these esters in NaHCO₃ at 37°. The homogenate (1 ml. of 40 mg./ml.) was diluted with water (5 ml.) and 0.3 ml. bromothymol blue (0.1%) added. The ester (1 ml.) was added, the mixture shaken, and the pH adjusted to neutral by cautious addition of NaOH solution (0.01 N). Enzyme and substrate blanks

were also set up. The time was noted, and the pH of the mixtures continuously adjusted by additions of 0.01N-NaOH from a micro-burette. The total volume of alkali was recorded at intervals. Each of the reaction mixtures in a run was matched against a colour standard consisting of 6 ml. 0.033M-phosphate pH 7.4 buffer + 1 ml. homogenate + 0.3 ml. bromothymol blue.

Phosphatase activity

Four types of phosphatase activity were studied—alkaline and acid phosphomonoesterase, inorganic pyrophosphatase and adenosinetriphosphatase (ATP-ase). Activities were measured at 37° by estimation of orthophosphate liberation, using as substrates sodium glycerophosphate, $\text{Na}_2\text{P}_2\text{O}_7$, or ATP. In the standard procedure the tissue suspension was added to 2 ml. Walpole acetate or Michaelis veronal buffer solution. (The pH values recorded were those of the final mixtures, including substrate and enzyme.) The total volume of the reaction mixture in each case, including activating substances where necessary, was 4 ml. Appropriate enzyme and substrate blanks were also set up. The conditions employed for the different types of phosphatase activity were as follows:

Alkaline phosphomonoesterase (pH 9.6). Tissue concentration was 4–7 mg./ml., substrate concentration 6–20 mM, and incubation period 1 hr. MgCl_2 (5 mM) was included as an activator.

Acid phosphomonoesterase (pH 5.2). Tissue concentration was 4–7 mg./ml., substrate concentration 6–18 mM, and incubation period 2 hr. MgCl_2 was omitted.

Pyrophosphatase (pH 7.4). Tissue concentration was 1.5–2.0 mg./ml., substrate concentration 1 mM, and incubation period 20–30 min. MgCl_2 (8 mM) and cysteine (CySH, 10 mM) were included as activators.

Adenosinetriphosphatase (pH 7.4). Tissue concentration was 1.5–2.0 mg./ml., substrate concentration 1.7–3.0 mM, and incubation period 15 min. MgCl_2 (4 mM) and CySH (10 mM) were included as activators.

Variations in the above conditions were made to establish optimal conditions for enzyme assay. In experiments where inhibitors were used, they were included in the incubation mixture prior to addition of substrate, except in the case of alcohol, where the conditions employed by Abul-Fadl & King (1949) were employed.

The tubes containing the reaction mixtures were shaken at 37° for the appropriate incubation period and then treated with 10% CCl_3COOH (2 ml.) and allowed to stand 20 min. before filtration. Orthophosphate was estimated in suitable portions (usually 3 ml.) of the filtrate by the method of Fiske & Subbarow (1925), the colours being read on the Spekker photoelectric absorptiometer, using Ilford orange filters. Results are expressed as μmol . substrate decomposed/30 min./g. wet wt. tissue. (In the case of ATP-ase, it was assumed that only one orthophosphate group was liberated during the short period of incubation employed.) It was confirmed that none of the substances used as inhibitors was able to interfere with the Fiske-Subbarow estimation under the conditions used.

Adenosinetriphosphatase. A commercial sample of the barium salt was washed with ice-cold pH 4.0 acetate buffer (0.1M) as recommended by Kornberg (1950) and converted to the sodium salt by the method of Bailey (1942). The concentration was checked by a 7 min. hydrolysis test.

Glycerophosphate. The sample used was found to contain 8.7% of the α - and 91.3% of the β -isomer, when analysed by the method described by Motzok & Wynne (1950).

RESULTS

Hydrolysis of aliphatic and choline esters

Initial rates of hydrolysis shown by a series of simple esters of primary alcohols, compared with tributyrin, are shown in Fig. 1. Only *n*-butyl *n*-propionate showed a hydrolysis rate higher than tributyrin, while the methyl, ethyl, *n*-propyl and *n*-butyl esters of *n*-butyric acid were all split at considerably lower rates. Rates of hydrolysis of

Fig. 1. Hydrolysis of aliphatic esters by rat adrenal cortex at 37° (measured manometrically). Tissue homogenate with 0.066% (v/v) ester. A, tributyrin; B, *n*-butyl acetate; C, *n*-butyl *n*-propionate; D, *n*-butyl butyrate; E, *n*-propyl butyrate; F, ethyl butyrate; G, methyl butyrate.

glyceryl acetates compared with tributyrin, measured by the titration technique, are illustrated in Fig. 2. The hydrolysis rates of monoacetin and diacetin were much lower than for triacetin, which itself showed a rate of hydrolysis roughly comparable with, though rather less than, that of tributyrin. In view of the relatively greater activity towards the triglycerides, the possibility of the enzyme system acting on natural fats such as triolein was examined. There was, however, no evidence of any measurable activity towards emulsified triolein, when tested by the methods described, or when the method of Willstätter, Waldschmidt-Leitz & Memmen (1923) was applied. It is evident that the lipolytic activity of the adrenal cortex is of a different character from that of the pancreas; it appears, in fact, to bear a closer resemblance to the plasma esterase studied by Adams & Whittaker (1949). The activity was proportional to tissue concentration over the range 1–7 mg./ml., so that accurate assays of activity levels are possible.

Compared with the hydrolysis of tributyrin the rates of hydrolysis of choline esters are low. A typical experiment gave activities of 8950 with tributyrin, 635 with ACh, 338 with MeCh, and 150 with BCh. The higher activity with MeCh compared

characterization of these esterase systems the influence of a number of substances on the activity was studied. Substances tested were the sodium salts of bile acids, which were found to activate pancreatic lipase (Holwerda, 1938); also benzaldehyde, sodium fluoride and copper sulphate, all of which inhibit pancreatic lipase (Weinstein & Wynne, 1935). In addition, it was of interest to test the effects of certain cholinesterase inhibitors, in view of the relatively greater activity of the enzyme towards MeCh, compared with BCh. Substances of this type studied were eserine, which inhibits both specific and non-specific cholinesterases (Hawkins & Mendel, 1946); the compound Nu 1250, i.e. the *N-p*-chlorophenyl-*N*-methylcarbamate of *m*-hydroxyphenyltrimethylammonium bromide, which is regarded as a selective inhibitor of specific cholinesterase (Hawkins & Mendel, 1949); and two compounds which show selective toxicity to the non-specific cholinesterase, i.e. the compounds Nu 683 (the dimethylcarbamate of 2-hydroxy-5-phenylbenzyltrimethylammonium bromide, Hawkins & Gunter, 1946) and 2987 R.P. (*N*-diethylaminoethylphenothiazine, Gordon, 1948). ACh only was used as substrate for cholinesterase in these experiments.

The results are seen in Table 1, and reveal several interesting features. The hydrolysis of tributyrin was strongly inhibited by the sodium salts of cholic, deoxycholic and tauroglycocholic acids, but only slightly affected by benzaldehyde—this contrasts with the behaviour of the lipase of pancreas, though the adrenal cortex enzyme is inhibited by sodium fluoride and by copper sulphate. None of the cholinesterase inhibitors produced any marked effect on the hydrolysis of tributyrin. Cholinesterase

Fig. 2. Hydrolysis of glycerides by rat adrenal cortex at 20° (measured titrimetrically). Tissue homogenate with 0.14% (v/v) ester. A, tributyrin; B, triacetin; C, diacetin; D, monoacetin.

with BCh has been previously noted for adrenal cortex by Sawyer & Everett (1947) and for whole adrenals by Ord & Thompson (1950). This observation would appear to place it in the 'specific' cholinesterase class, according to the nomenclature of Mendel & Rudney (1943).

Effects of inhibitors. To assist in the further

Table 1. *Effects of various substances on aliphatic esterase and cholinesterase activities of rat adrenal cortex at 37°*

Added substance	Concn. (mm)*	Inhibition of activity (%)	
		Aliphatic esterase (0.066% tributyrin as substrate)	Cholinesterase (12 mm-ACh as substrate)
Sodium cholate	7	58	14
Sodium deoxycholate	2.4	91	2
Sodium tauroglycocholate	3	89	0
Benzaldehyde	0.17% (v/v)	12	18
CuSO ₄	0.1	94	10
NaF	40	50	54
2987 R.P.	0.022	—	52
2987 R.P.	0.1	—	72
2987 R.P.	0.3	0	—
Nu 1250	5 × 10 ⁻³	4	24†
Nu 683	3 × 10 ⁻³	5	93†
Nu 1250	5 × 10 ⁻⁵	—	2
Nu 683	5 × 10 ⁻⁵	—	48
Eserine sulphate	0.1	9	72

* Except where stated otherwise. † Using 6 mm-ACh.

activity, on the other hand, was uninfluenced by the substances which inhibited tributyrin hydrolysis, with the exception of sodium fluoride. The effects of the cholinesterase inhibitors on cholinesterase activity were somewhat unexpected. 2987 R.P. inhibits strongly in a concentration of 0.022 mM, though concentrations up to 0.5 mM were previously found ineffective against specific cholinesterase in brain or plasma (Gordon, 1948). Only moderate inhibition was caused by 0.005 mM-Nu 1250, which in 0.001 mM concentration almost completely inhibits specific cholinesterase in brain and erythrocytes (Hawkins & Mendel, 1949). Nu 683, however, in similar concentrations, produced marked inhibition. These findings make it difficult to classify adrenal cortical cholinesterase on the basis of Mendel & Rudney's (1943) system. At the same time, it is clear that the choline esterase and aliphatic esterase systems are distinct from each other.

Phosphatase activities

Effect of pH on activity. Phosphomonoesterase activities at various pH values, with glycerophosphate as substrate, are indicated in Fig. 3. This type

Fig. 3. Effect of pH on phosphomonoesterase activity of rat adrenal cortex at 37°. Tissue homogenate with 6 mM-sodium glycerophosphate. Mg^{++} absent.

of activity shows the usual characteristic pH optima in the acid and alkaline regions, as in most animal tissues. Only one optimum pH value was found in the acid region; there was no evidence of a second acid optimum, as has been found for erythrocytes (Roche, Thoai & Baudouin, 1942; Abul-Fadl & King, 1949). With sodium pyrophosphate or ATP as substrates, the optimum pH was 7.4 (Fig. 4). The presence of a strong ATP-ase activity in adrenals has already been reported (Zeller, 1948).

Influence of substrate concentration. The effects of substrate concentration are seen in Figs. 5 and 6. Well defined optimal substrate concentrations were obtained with pyrophosphatase (0.3 mM) and ATP-ase (1.5 mM). The effects with acid and alkaline phosphomonoesterases were not so clear-cut; on

the basis of these results, however, a substrate concentration of 18–20 mM provides near-optimal conditions.

Fig. 4. Effect of pH on phosphatase activity of rat adrenal cortex at 37°. A, 1 mM- $Na_4P_2O_7$ + 8 mM- Mg^{++} ; B, 1.7 mM-ATP + 4 mM- Mg^{++} .

Fig. 5. Effect of substrate concentration on phosphomonoesterase activity of rat adrenal cortex at 37°. Sodium glycerophosphate as substrate. A, pH 9.6; B, pH 5.2.

Fig. 6. Effect of substrate concentration on phosphatase activity of rat adrenal cortex at 37° and pH 7.4. A, $Na_4P_2O_7$ with 20 mM- Mg^{++} . B, ATP with 4 mM- Mg^{++} .

Activation by Mg^{++} . The behaviour of the different systems towards magnesium is similar to that found in other tissues—alkaline phosphatase is activated appreciably by Mg^{++} in concentrations of 1–10 mM, but acid phosphatase is not perceptibly influenced. Mg^{++} is essential for the activity of pyrophosphatase and shows an optimal effect at 4 mM. ATP-ase is markedly stimulated by Mg^{++} , the effect being maximal at 3–4 mM, but declining again at higher concentrations. Ca^{++} also activates ATP-ase, but not to the same extent as Mg^{++} .

Fig. 7. Effects of cysteine on phosphatase activities of rat adrenal cortex at 37°. A, 12 mM-sodium glycerophosphate + 5 mM- Mg^{++} , pH 9.6; B, 18 mM-sodium glycerophosphate, pH 5.2; C, 1 mM- $Na_4P_2O_7$ + 8 mM- Mg^{++} , pH 7.4; D, 1.9 mM-ATP + 4 mM- Mg^{++} , pH 7.4.

Effects of cysteine. CySH was found to bring about definite activation of both pyrophosphatase and ATP-ase (Fig. 7), the maximum effect being produced with a concentration of 5–12 mM. Alkaline phosphatase, like that of some other tissues, was strongly inhibited by CySH, but acid phosphatase was uninfluenced.

Effects of tissue concentration. Fig. 8 shows the effects of tissue concentration. Good proportionality was obtained with the phosphomonoesterases at concentrations up to 8.5 mg./ml. for alkaline phosphatase and 7.5 mg./ml. for acid phosphatase. With pyrophosphatase and ATP-ase the inclusion of CySH as a co-activator is important from the point of view of enzyme assay; in its absence, a non-linear tissue concentration curve was obtained, but the linear relationship was restored when CySH was present. This phenomenon is possibly due to a protective action against partial deactivation by oxidation.

Progress of hydrolysis. Rates of hydrolysis remain constant in each case for periods of time well outside the selected times used in the assay. For ATP-ase, the relationship holds up to 30% decomposition, and for pyrophosphatase up to 60% decomposition, of substrate. The phosphomonoesterase activities, though not studied up to the maximum period of

constant rate, are constant up to a stage which covers the assay conditions.

Effects of various substances on acid phosphatase. Since the acid phosphatases of different tissues respond differently to certain inhibitors, it was of interest to test the action of some of these inhibitors on the adrenal cortex enzyme. The substances tried were selected from those used by Abul-Fadl & King (1949) in the differential characterization of plasma

Fig. 8. Effects of tissue concentration on phosphatase activity of rat adrenal cortex at 37°. A, 19 mM-sodium glycerophosphate, pH 5.2; B, 6 mM-sodium glycerophosphate, pH 9.6; C, 1 mM- $Na_4P_2O_7$ in presence of 8 mM- Mg^{++} + 10 mM-CySH, pH 7.4; D, 3 mM-ATP in presence of 4 mM- Mg^{++} + 10 mM-CySH, pH 7.4.

acid phosphatases. Results are shown in Table 2. The behaviour was to some extent similar to that of the red-cell acid phosphatase. Ethanol and formaldehyde inhibited substantially; copper sulphate

Table 2. *Effects of various substances on acid phosphatase activity of rat adrenal cortex at 37°*

(Acetate buffer, pH 5.2. Sodium glycerophosphate (18 mM) as substrate. In the case of ethanol the tissue was pre-incubated with 0.4 vol. ethanol before making up the incubation mixture; otherwise the inhibitors were added directly to the incubation mixture.)

Added substance	Concn. (mM)	Inhibition (%)
$FeSO_4$	10	0
$CuSO_4$	0.2	86
Formaldehyde	170	30
Formaldehyde	600	73
Ethanol	—	68
Sodium DL-tartrate	—	91

in low concentration also caused strong inhibition, but ferrous sulphate was without effect. On the other hand, the enzyme was strongly inhibited by DL-tartrate, which does not affect the red-cell enzyme (Abul-Fadl & King, 1949).

DISCUSSION

The investigations indicate the general behaviour of the esterase and phosphatase reactions of the adrenal cortex, though the role of these systems in adrenal cortical function must clearly require much more detailed and extensive studies.

It seems doubtful whether the term 'lipase' can be applied to the tributyrinase action of adrenal cortex homogenates, in view of the apparent inactivity towards triolein, and the different response shown to bile salts as compared with pancreatic lipase. Since the earlier reports on the existence of an adrenal cortical lipase were based on its activity towards tributyrin, the point only involves a question of nomenclature. Since the enzyme bears a closer resemblance, with respect to substrate specificity, to the esterase of human plasma (cf. Adams & Whittaker, 1949), the term 'aliphatic esterase' would be the more appropriate.

The influence of various inhibitors on the aliphatic esterase and cholinesterase activities suggests that choline esters are hydrolysed by an enzyme system which is distinct from the aliphatic esterase, though the latter is present in so much greater strength. The results on cholinesterase activity are not easy to interpret. Although acetyl- β -methylcholine is attacked much more readily than benzoylcholine, the designation of 'true' or 'specific' cholinesterase is contra-indicated by the anomalous behaviour towards the selective inhibitors used. Further light on this matter would perhaps be thrown by experiments using a wider range of inhibitors. It seems possible in any case that cholinesterase plays a less important role in the cortex than in the medulla. It has been reported (Lange-mann, 1942) that the enzyme is present in much greater strength in the medulla than in the cortex; this was confirmed during the course of the present work by a few rough measurements (not reported) on pooled separated medullae, which indicated reaction rates approximately three times as high as for cortex. This distribution may be connected with the relative distribution of post-ganglionic sympathetic nerve fibres in the gland.

The properties of the adrenal cortical phosphatases, especially in regard to their pH optima and

the manner in which they are influenced by magnesium ions, are broadly similar to those of other tissues. ATP-ase and pyrophosphatase show characteristics similar to those of the corresponding water-extractable enzymes of other animal tissues; ATP-ase is activated by calcium and magnesium ions (cf. Frank, Lipschitz & Barth, 1950), the latter being the more effective, while pyrophosphatase is only active in presence of magnesium ions (cf. Naganna & Menon, 1948; Gordon, 1950).

At the present stage, perhaps the most important result arising from the investigations just described is the demonstration that, with the exception of cholinesterase, the levels of activity of all the enzyme systems concerned may be assayed in quantities of cortical tissue obtainable from the adrenals of an individual rat, so that the investigation of changes taking place under various abnormal conditions may be readily undertaken.

SUMMARY

1. Homogenates of demedullated rat adrenals show greater esterase activity with triacetin and tributyrin than with aliphatic esters of primary alcohols, and are inactive towards triolein. Choline esters are hydrolysed much less readily than tributyrin.

2. Bile salts and copper sulphate inhibit the aliphatic esterase. Eserine, the dimethylcarbamate of 2-hydroxy-5-phenylbenzyltrimethylammonium bromide (Nu 683) and *N*-diethylaminoethylphenothiazine (2987 R.P.), but not the *N*-*p*-chlorophenyl-*N*-methylcarbamate of *m*-hydroxyphenyltrimethylammonium bromide (Nu 1250), inhibit the cholinesterase.

3. The phosphomonoesterase, pyrophosphatase and adenosinetriphosphatase activities of such homogenates have also been studied. Acid phosphatase is inhibited by copper sulphate, formaldehyde, ethanol and DL-tartrate, but not by ferrous sulphate.

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The Oxidation of Myoglobin to Metmyoglobin by Oxygen. 1

By P. GEORGE AND C. J. STRATMANN

Department of Colloid Science, University of Cambridge

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The study of the oxidation of haemoglobin and myoglobin to methaemoglobin and metmyoglobin by molecular oxygen is important in understanding the stability of oxyhaemoglobin and oxymyoglobin.

Brooks (1931, 1935) investigated the oxidation of haemoglobin (Hb) to methaemoglobin and found that under constant conditions of oxygen pressure, pH, salt concentration and temperature the reaction was first order with respect to the concentration of unoxidized haemoglobin, and that the rate of oxidation had a well defined maximum value at a low oxygen pressure (about 20 mm. at 30°).

Legge (1942) suggested the spontaneous decomposition of one of the oxygenation intermediates $\text{Hb}_4(\text{O}_2)_2$ as a reaction mechanism to account for these results. Later Lemberg & Legge (1949) amplified this mechanism by assuming the participation of two oxidizable XH_2 groups on the protein to make possible an intramolecular reaction fitting the stoicheiometric relation:

George (1949) put forward an alternative representation of the kinetics and discussed a possible intermolecular free radical mechanism which avoids some difficulties inherent in an intramolecular mechanism (George, 1952).

An investigation of the oxidation of myoglobin should help in deciding between mechanisms of this kind, for, since myoglobin has only one haem group per molecule, a reaction of type *A* occurring in this system should necessarily be second order.

This paper deals with the general chemistry of the reaction—choleoglobin and denatured protein formation—the overall stoicheiometry and the results of a kinetic study to establish this point.

In this paper the symbols Mb, MbO_2 , MetMb are used to represent reduced myoglobin, oxymyoglobin and metmyoglobin respectively. Similarly, in the case of haemoglobin, the corresponding symbols Hb, HbO_2 and MetHb are used.

MATERIALS AND METHODS

Preparation of pure, recrystallized equine metmyoglobin. The method employed is based on that of Theorell (1932) and Lawrie (1951). Four hearts were usually processed. After machine-mincing, the muscle tissue was extracted with water, the solution neutralized and treated with 1/5 of its volume of saturated, basic lead acetate to remove muscle globulins and other impurities. After centrifugation, the solution was again neutralized and sufficient solid Na_2HPO_4 to remove excess lead was added. The pH was once more returned to 7.0 by addition of solid NaH_2PO_4 and the solution was centrifuged. It was found particularly important to adjust the pH to 7.0 at the points indicated above in order to obtain the maximum yield. It was also desirable to keep the temperature as near 0° as possible throughout the preparation. The MetMb solution was further purified by crystallization, induced by saturation of the solution with ammonium sulphate, followed by solution in 3 M-phosphate buffer of pH 6.8. This treatment separates the MetMb from any traces of haemoglobin which may be present since haemoglobin is almost completely insoluble in phosphate buffer of this molarity (Morgan, 1935).

Lastly, the material was dissolved in the desired buffer solution (see below), traces of $(\text{NH}_4)_2\text{SO}_4$ were removed by