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Determination of the Arginase Activities of Homogenates of Liver and Mammary Gland: Effects of pH and Substrate Concentration and Especially of Activation by Divalent Metal Ions

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In our previous studies of the arginase activities of certain tissues of the rat (Folley & Greenbaum, 1946, 1947a) we used reaction periods of 30 min. and 20 hr. for homogenates of liver and mammary gland respectively, and assessed the activities of the homogenates in terms of our 30 min. or 20 hr. arginase units from enzyme calibration curves. Arginase undergoes inactivation at alkaline pH values (Hunter & Morrell, 1933a), a factor particularly serious in methods involving long reaction periods. Nevertheless, we feel confident that our previous findings regarding changes in the arginase activity of the mammary gland under various conditions are valid, since all values were read from a calibration curve which not only was reproducible with different mammary-gland homogenates, but also agreed with a curve obtained for one of these homogenates in the presence of Co^{++} (see Fig. 1). The significance of the latter agreement is not lessened by our present belief (see later section) that the effect of Co^{++} under these conditions was mainly to inhibit reversible inactivation due to dissociation of the enzyme system.

However, the use of different units for liver and mammary-gland homogenates was hardly practicable for further studies involving comparison of arginase activities of these tissues in the same animal, since the comparison can only be made if the quantitative relationship between the two units is known, and while the highly active liver homogenates can be assayed by both methods, the high dilution necessary for the 20 hr. method favours dissociation of the
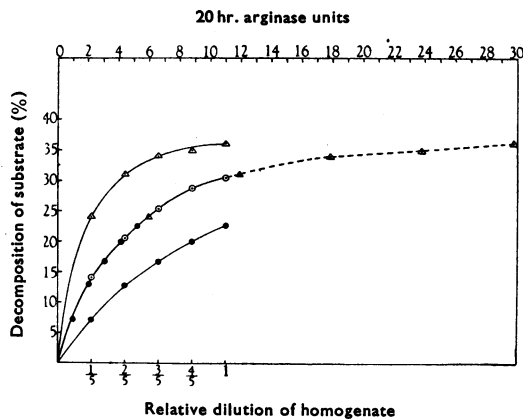

Fig. 1. Calibration curve (circles) for the determination of arginase in mammary-gland homogenates by the method of Folley & Greenbaum (1947a). The bottom curve (black dots) represents the arginase activities of various dilutions of an homogenate less active than the one used for the construction of the calibration curve; the top curve (triangles) represents the arginase activities of corresponding dilutions of the same homogenate in the presence of Co^{++}. The upper horizontal scale (20 hr. arginase units) refers only to the central curve, to which the other two sets of points have been fitted by appropriate adjustment of their horizontal scales, i.e. transformation of their abscissae into 20 hr. arginase units. It will be seen that the bottom curve gives a very satisfactory fit to the standard calibration curve, while the greater part of the top curve provides an extension (broken line) of it.

metal-enzyme with consequent loss of activity at the outset. In addition, we wished to investigate the activation of the arginase in mammary-gland and liver homogenates by certain divalent metal ions. Here again, methods involving prolonged hydrolysis are unsuitable, since, if the supposed activating agent merely protects the enzyme against inactivation, it will apparently increase its activity. For these two purposes we require, therefore, a method applicable to homogenates with widely different activities, yet employing so short a reaction period that approximate initial velocities are measured.

The sensitive colorimetric reagent for urea described by Archibald (1945), and applied by Van Slyke & Archibald (1946) to the determination of arginase activity, was the best basis for a method allowing the estimation of approximate initial reaction velocities for tissues with such widely different activities as liver and mammary gland. The advantages of a colorimetric method for carrying out a number of determinations simultaneously are obvious, and it is better to determine liberated urea rather than residual arginine, not only because of the remote possibility that arginine may be decomposed by enzymes other than arginase without the liberation of urea (see Van Slyke & Archibald, 1946), but also because the relative changes in arginine, for short reaction periods, are very small. Whilst we have no reason to believe that our earlier experiments (Folley & Greenbaum, 1947a), in which considerable proportions of the substrate were hydrolyzed, were affected by errors due to either of these factors, we have now adapted the photometric method of Van Slyke & Archibald (1946) for use with our tissue homogenates.

The pH-activity and substrate concentration-activity relationships under our conditions have been determined, and also the activation of the arginase of homogenates by Mn^{++} and Co^{++}. The results show that Mn^{++} is a better activator than Co^{++} and that the enzyme exists in the tissues as an easily, but reversibly, dissociable manganese-protein complex which may not always be fully activated.

EXPERIMENTAL

Tissue homogenates. Homogenates of liver and of lactating mammary gland of rats were prepared as described previously (Folley & Greenbaum, 1947a) with the homogenizer of Folley & Watson (1948). Tissues were homogenized for 5 min. at room temperature, liver with 9 vol. of saline (0·9% NaCl) and mammary gland with 19 vol., the larger dilution being necessitated by the limited amount of gland usually available and the dimensions of the homogenizer. The amount of mammary tissue available from one rat is restricted since only the 'abdominal' (i.e. the two abdominal and four inguinal) glands, and not the thoracic glands, which are much more richly interlaced with strands of muscle and connective tissue, were used. The tissues were dissected out and homogenized as rapidly as possible, and the enzyme determinations always started within 15 min. of the death of the rat, which was killed by dislocation of the spine.

In much of this work it was found convenient to carry out activity determinations on liver homogenates further

diluted 1:20 with saline (1 ml.≡5 mg. fresh tissue), whilst mammary gland homogenates were used undiluted (1 ml.≡ 50 mg. fresh tissue). For activity determinations involving only 1 min. hydrolysis, liver homogenates were further diluted only 1:8 (1 ml.≡12·5 mg. fresh tissue).

Preparation of reagents. The urea reagent of Archibald (1945) (α-*iso*nitrosopropiophenone) was synthesized by the method of Claisen & Manasse (1889). Yield, c. 75%; m.p. after recrystallization from hot water, 114–115° (uncorr.). The substrate used throughout was L-arginine monohydrochloride, prepared from gelatin by the method (1) of Brand & Sandberg (1932).

Determination of enzyme activity. The method finally adopted, on the basis of the present results, for routine determination of the arginase activity of tissue homogenates differs from that of Van Slyke & Archibald (1946) mainly in that we use a higher reaction temperature (37°) and a lower substrate concentration (0·227M), the latter being just sufficient to give the maximum initial velocity at our (optimal) pH of 9·45.

The reaction mixture consists of 2·5 ml. of 8·0% (w/v) L-arginine monohydrochloride (titrated to pH 9·45 at 37°), 1·25 ml. of glycine buffer (pH 9·45 at 37°) and 0·3 ml. of a suitably diluted homogenate. The buffer-substrate is pre-heated to 37° in the thermostat, whilst the homogenate, which is best kept at room temperature if it contains no added Mn^{++}, is diluted if necessary to the required degree with saline at 37° immediately before mixing with the buffer-substrate. The reaction is stopped after a given time (usually 5 min.) by the addition of 0·95 ml. of freshly prepared 30% (w/v) HPO_3, and the precipitate of protein filtered off after 30 min. at room temperature. Thereafter the colour is developed as described by Van Slyke & Archibald (1946), 2·0 ml. of filtrate being used and the colour measured in a Miller photoelectric absorptiometer (Ilford spectrum green filter no. 604), usually with a 1 cm. cell or, with intense colours, a 0·5 cm. cell. An enzyme blank is set up and treated in exactly the same way, except that the HPO_3 is added before the homogenate. The arginase unit is now defined as that quantity of enzyme which, under the conditions specified above, liberates 1 μmol. of urea (0·06 mg.)/min. at 37° and pH 9·45 from L-arginine in 0·227M concentration.

If the fully activated arginase activity is required, sufficient $MnSO_4$ solution to give a final concentration of 2 mg. Mn^{++}/ml. of homogenate is pipetted into a test tube and taken to dryness. The requisite amount of homogenate, if necessary (e.g. with liver homogenates) diluted ready for enzyme determination, is then added and incubated at 37° for 1 hr. These conditions have been found to give full activation (see later section). Alternatively, the tissue can be homogenized with saline containing the correct concentration of Mn^{++}, and any subsequent necessary dilution carried out with saline containing 2 mg. Mn^{++}/ml. In the presence of Mn^{++} (or Co^{++}) under these conditions, a fine flocculent precipitate is formed in the homogenate, removal of which would almost certainly result in loss of enzyme activity (see, e.g., Hunter & Downs, 1944). This precipitate can be uniformly dispersed by vigorous shaking just before pipetting.

We recommend a 5 min. hydrolysis period for routine use, except for relatively inactive homogenates, though in the present work we have often used 10 min. Periods longer than 10 min. are neither necessary with this technique, nor desirable, if inactivation of the enzyme during the run is to

be minimized. The method is, however, sufficiently sensitive to allow further reduction to 1 min. with liver homogenates if desired; however, beyond this point random errors in measurement of time assume undue importance.

Investigations described later show that with reaction periods of either 5 or 10 min., if more than about 1 % of the substrate is hydrolyzed, the observed urea production is no longer linearly related to the enzyme concentration and an enzyme calibration curve must be used, whereas below this limit the arginase units can be arithmetically calculated. The reaction period (max. 10 min.) and the dilution of the homogenate should therefore be chosen accordingly. It is recommended that for 5 min. reaction periods no more than 300 units of enzyme/l. ($\equiv$0·66 % hydrolysis in 5 min.) be taken.

able, and such effects are almost certainly negligible. Separate standardization curves for experiments in which Mn^{++} (or Co^{++}) are added to the homogenates are necessary (Mn^{++} intensifies the colour), and were constructed in a similar way. All our galvanometer curves were linear.

Van Slyke & Archibald (1946) imply that the pH (9·5), which they recommend for routine arginase activity determinations, is slightly on the acid side of the optimum for liver enzyme, and they give reasons why they prefer to work at this pH rather than at pH 9·7, at which, they state, arginase may be slightly more active. The pH we specify for
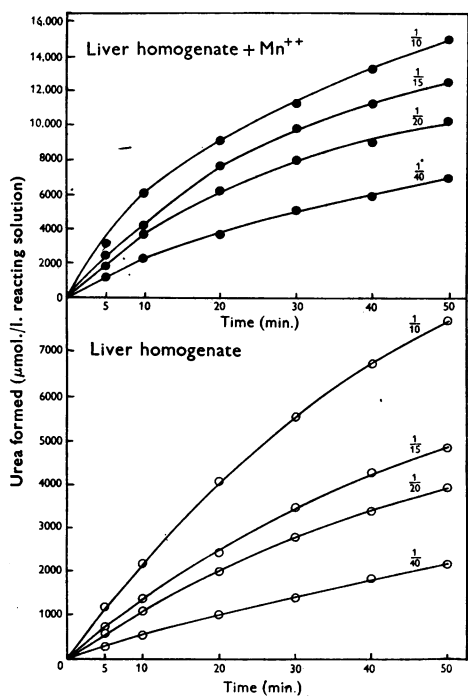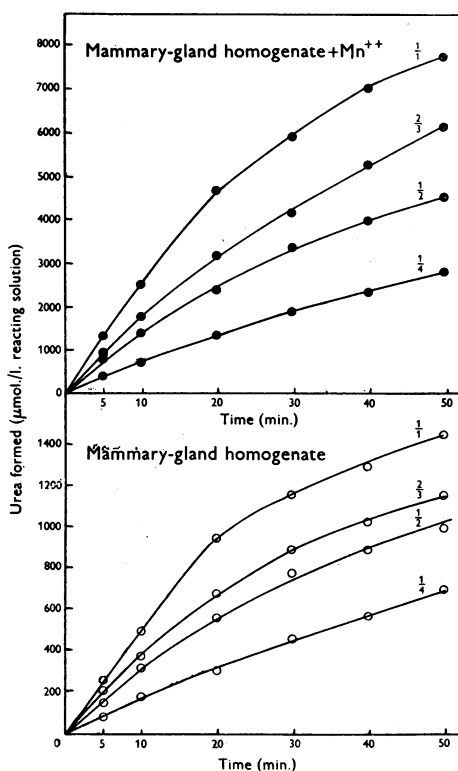

Fig. 2. Arginase reaction-velocity curves for various dilutions of a rat-liver homogenate, with and without added Mn^{++}. The reaction conditions were as follows: temperature 37°, pH 9·45, substrate concentration 0·227 M. The dilution of the original homogenate used for each experiment is indicated near the relevant curve.

Fig. 3. Arginase reaction-velocity curves for various dilutions of a rat mammary-gland homogenate, with and without added Mn^{++}. The reaction conditions were as follows: temperature 37°, pH 9·45, substrate concentration 0·227 M. The dilution of the original homogenate used for each experiment is indicated near the relevant curve.

According to Van Slyke & Archibald (1946) arginine diminishes the colour arising from the reaction between urea and α-isonitrosopropiophenone, and this we have confirmed. It is accordingly necessary to correct for this and any interference with the colour development which may be due to other components of the hydrolysates. Accordingly, galvanometer readings are converted into mg. urea by reference to a standard curve constructed from measurements on a series of mock hydrolysates containing known amounts of urea and appropriate amounts of all other components. Correction for the possible interfering effects of substances present in the tissue homogenates is obviously impractic-

routine determinations is slightly more acid than that used by Van Slyke & Archibald, but is, nevertheless, the optimum for the enzyme in both liver and mammary-gland homogenates. However, the increased stability of the enzyme on the acid side of the optimum (Hunter & Morrell, 1933a) made it desirable to carry out parallel studies at lower pH, particularly since a number of authors (Hunter & Dauphinee, 1930; Lightbody, 1938; Kochakian, 1944; Hunter & Downs, 1944; Folley & Greenbaum, 1946, 1947a) have described arginase activity methods involving hydrolysis on the acid side of the pH optimum. For activity determinations at pH 9·1, the lower pH adopted, we used

substrate in 0·071 M final concentration, which our investigations (see later section) indicated as the optimal concentration at this pH.

Throughout, in investigating the effect of changes in various conditions on arginase activity, the hydrolyses were carried out basically as above, but with appropriate modifications according to the factor under study.

RESULTS AND DISCUSSION

Velocity of arginase action

Reaction-velocity curves, determined at pH 9·45 and in presence of 0·227 M-substrate with and without added Mn^{++}, for various dilutions of rat-liver and mammary-gland homogenates respectively, are given in Figs. 2 and 3. In neither case does any
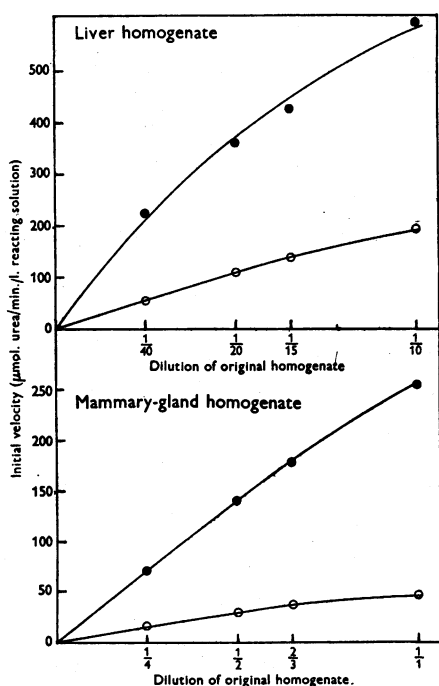

Fig. 4. Relation between arginase concentration and initial reaction velocity, calculated from the urea production in the first 10 min., for homogenates of rat liver and mammary gland, with (black dots) and without (circles) added Mn^{++}. All experiments at 37° and pH 9·45; substrate concentration 0·227 M.

reaction rate depart significantly from linearity over the first 10 min., though inhibitory effects, probably due to progressive inactivation of the enzyme and to the accumulation of the inhibitory reaction product L-ornithine (Edlbacher & Zeller, 1936), become apparent soon after. In the runs with activated enzyme any progressive inactivation which occurred may be assumed to be due to the high alkalinity (see

Hunter & Morrell, 1933a) and was presumably irreversible; the non-activated enzyme would also be liable to reversible inactivation due to gradual dissociation of the metal-enzyme (see later section).

Thus for reaction periods up to about 10 min. the amount of urea produced in a given time is proportional to the initial reaction velocity. Since the enzyme was in the presence of excess substrate, it may be anticipated that initial reaction velocities will be proportional to enzyme concentrations, so that for reaction periods up to 10 min. the amounts of urea produced should be convertible by simple calculation into arginase units without reference to an enzyme calibration curve, as was found by Van Slyke & Archibald (1946) under their conditions. This is indeed the case, provided no more than about 1·0 % of the initial substrate is hydrolyzed (Fig. 4); the only curve which deviates appreciably from linearity is that for Mn^{++}-activated liver homogenate. Similar results were obtained at pH 9·1 in presence of 0·071 M-substrate, though in this case the upper limit of substrate decomposition for preservation of an approximately linear relation between enzyme concentration and urea production was 5 %.

pH-Reaction velocity relationships

In investigating pH-activity relationships the enzyme activities were determined by the standard method except that appropriate glycine buffers were used, and the substrate solutions titrated beforehand to the requisite pH. For each pH value an extra reaction tube was provided solely for pH determination at the beginning and end of the hydrolysis. In no case was any appreciable change in pH observed during the reaction. All pH values were measured by glass electrode at 37°.

Typical curves for homogenates of liver and mammary gland from the same rat, determined in presence of 0·227 M-substrate, are shown in Figs. 5 and 6, the ordinates in Fig. 6 representing initial reaction velocities expressed as percentages of the maximum. These curves cover a narrower pH range (particularly on the acid side of the optimum), but with smaller intervals than those commonly given in the literature so that they allow of closer definition of the pH optima than usual.

The pH optima (substrate concentration 0·227 M) for non-activated homogenates of both tissues were essentially the same (pH 9·43–9·45). Similar optima for liver arginase were reported by Felix & Schneider (1938) and for both liver and jack-bean enzyme by Damodaran & Narayanan (1940). For liver arginase, more alkaline optima were given by Edlbacher & Bonem (1925) (pH 9·5–9·8), Hunter & Morrell (1933b) (pH 9·8) and Mohamed & Greenberg (1945) (pH 10·0); and more acid optima by Hino (1926) (pH 7·3–7·5), Edlbacher & Simons (1927) (pH 9·0) and Hellerman & Stock (1938) (pH 8·0). The latter

value may be compared with the value pH 9·0 given by Stock, Perkins & Hellerman (1938) for jack-bean arginase. Most of the above mentioned studies were concerned with glycerol extracts of liver or with partially purified liver extracts, but differences among these results, and between many of them and our own, may result from the dependence of apparent pH optima on many factors; hence there seems no reason to conclude that the pH-activity relationship for liver and mammary-gland homogenates differs in any important respect from that of purified preparations.
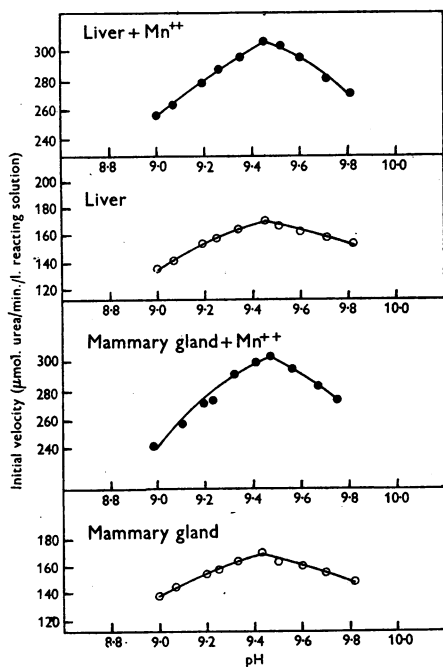

Fig. 5. Activity-pH curves for arginase in homogenates of rat liver and mammary gland, with (black dots) and without (circles) added Mn^{++}. All experiments at 37° and in presence of 0·227M-substrate. Reaction time 5 min.

Under our conditions, the pH optimum was unchanged by maximal activation with Mn^{++} (Figs. 5 and 6), and in fact, with the method of plotting used in Fig. 6, the points corresponding to activated and non-activated homogenates of both tissues fall on the same curve. In this respect tissue homogenates appear to differ from liver extracts or crude extracts of jack bean; for appreciable shifts of the pH optimum to more acid values in the presence of activating divalent cations have been reported by Hellerman & Stock (1938), Stock et al. (1938), Damodaran & Narayanan (1940) and Mohamed & Greenberg (1945), whilst Hellerman & Stock (1938) found that, though Ni^{++} and Co^{++} moved the optimum in the acid direction, Mn^{++} had the opposite effect. It is tempting, but perhaps unjustifiable at this stage, to conclude that since activation with Mn^{++} does not affect the pH optimum the behaviour of the enzyme in our homogenates is more 'natural' than that in purified enzyme preparations. In any event, the fact that full activation causes no appreciable change in pH optimum under our conditions facilitates the development of an arginase assay applicable to activated and non-activated homogenates alike.
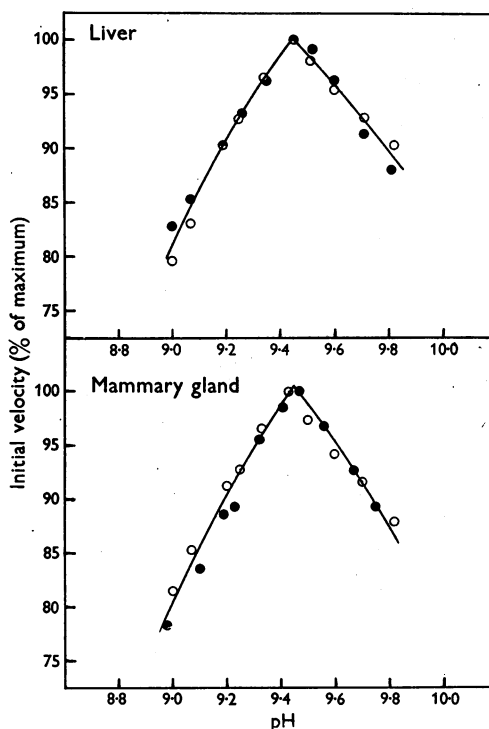

Fig. 6. Activity-pH curves for arginase in homogenates of rat liver and mammary gland, with (black dots) and without (circles) added Mn^{++}. All experiments at 37° in presence of 0·227M-substrate. Reaction time 5 min. The ordinates represent initial reaction velocities expressed as percentages of the initial velocity at the optimum pH.

The pH-activity relationships in the presence of 0·071M-substrate are shown in Fig. 7, in which the ordinates represent percentages of the maximum reaction velocity. With mammary-gland homogenates activation with Mn^{++} again had no effect on the pH optimum which, moreover, was unaffected by the change in substrate concentration. The pH optimum for the non-activated liver homogenate was now, however, slightly more acid (approx. pH 9·35), so that there is some indication in this case of a slight shift of the optimum (0·1 pH) towards the alkaline side in the presence of Mn^{++}.

The fact that an approximately threefold decrease in the substrate concentration had no appreciable effect on the pH optima is somewhat surprising, since there is evidence that K_m for arginase changes with pH (Greenberg & Mohamed, 1945). As will be shown in the next section, the lower of the two substrate concentrations used is optimal at pH 9·1 (inhibition occurring as the substrate concentration
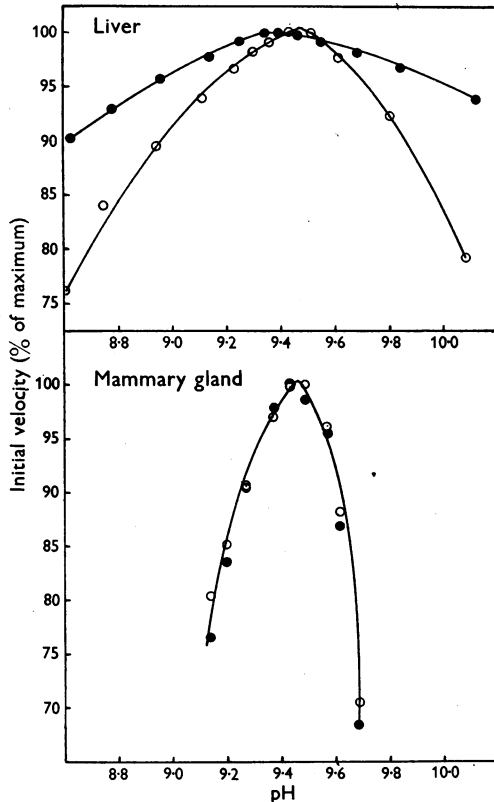
The pH-activity relationships for the arginase in tissue homogenates, at any rate in presence of excess substrate, are fairly critical. Thus at pH 9·1 and in presence of 0·227 M-substrate, the velocity is only about 85 % of that at pH 9·45. Since the curvature of pH-activity curves may well vary among homogenates from different animals, or homogenates from different tissues of the same animal (e.g. Fig. 7), tissue-arginase assays for purposes of quantitative comparison should be carried out at the optimal pH and with adequate buffering to prevent pH changes during the reaction. Furthermore, methods which involve hydrolysis on the acid side of the pH optimum should be used with caution.

Substrate concentration-reaction velocity relationships

Reaction velocity-substrate concentration curves, determined at pH 9·45, are shown in Fig. 8. In these experiments it was necessary to correct for variable

Fig. 7. Activity-pH curves for arginase in homogenates of rat liver and mammary gland, with (black dots) and without (circles) added Mn++. All experiments at 37° and in presence of 0·071 M-substrate. Reaction time 10 min. The ordinates represent initial reaction velocities expressed as percentages of the initial velocity at the optimum pH.

Fig. 8. Relation between substrate concentration and enzyme activity for arginase in homogenates of liver and mammary gland, with (black dots) and without (circles) added Mn++. All experiments at 37° and pH 9·45. Reaction time 10 min.

is increased), whilst at pH 9·45 the velocity in presence of 0·071 M-substrate is considerably less than the maximal. Thus it would be expected that, even if there is no shift in the optimal pH, the pH-activity curves at the lower substrate concentration would show appreciably less curvature than those at the higher substrate concentration. This is the case for the liver homogenates, but rather surprisingly the reverse holds for mammary-gland homogenates (cf. Figs. 6 and 7).

effects on the final urea colour of widely different arginine concentrations. Each solution, before the colour was measured, was therefore brought to a standard arginine concentration by suitable dilution with water or an appropriate arginine solution. There

was of course still some possible error since the amounts of urea, L-arginine and L-ornithine in many of these final solutions were no longer equivalent, as the galvanometer calibration curves require them to be. However, this residual error may be assumed to be too small to justify the amount of labour necessary to eliminate it.

The curves in Fig. 8 resemble that given by Van Slyke & Archibald (1946) in that there is no sign of inhibition at high values of substrate concentration (S), but they differ from it in reaching a virtual maximum or saturation value in the neighbourhood of $S = 0.22$ M, while the curve of Van Slyke & Archibald continues, after the initial rapid increase, to rise slowly up to $S = 0.4$ M. The highest maximal velocity was attained by activated liver homogenate (Fig. 8), and corresponds to an enzyme concentration of approximately 460 units/l. It may therefore be concluded that, in the presence of 0.22 M-substrate, enzyme concentrations at least up to this value are fully saturated in the initial stages of the reaction. We were thus led to specify a final substrate concentration of 0.227 M for our routine method of arginase activity determination, as being sufficient to give a maximum velocity with concentrations of enzyme likely to be encountered under our conditions, while corresponding to a conveniently prepared substrate solution (8 % w/v as monohydrochloride).

A very different type of activity-S relationship was revealed in the experiments at pH 9·1 (Fig. 9). Inhibition was now observed with high substrate concentrations so that the curves show definite optima at a substrate concentration (0.071 M) which is the same for all. It is evident that, for arginase activity determinations on the acid side of the optimal pH, considerably lower substrate concentrations should be used than for methods employing the optimal pH.

The change with pH in the type of activity-substrate concentration relationship exhibited by arginase in tissue homogenates is of interest, but no explanation will be attempted here. Damodaran & Narayanan (1940), studying liver arginase, also found some indication of inhibition with increasing substrate concentration. Their optimal substrate concentration was given as 0.067 M, a value not very different from ours at pH 9·1; but a puzzling feature is that their results were obtained at pH 9·4, which is not far from our optimal pH at which no inhibition at high substrate concentration occurs. No such inhibition is apparent in the results of Greenberg & Mohamed (1945), but their studies were not extended into a range of sufficiently high substrate concentration for inhibition to occur.

Enzyme inhibition by excessive substrate concentrations is a fairly common phenomenon (see Haldane, 1930; Lineweaver & Burk, 1934). Folley &

Kay (1935) observed it with unfractionated preparations of mammary-gland and kidney alkaline phosphatase, and they found that their results could be fitted passably well by Haldane's (1930) equation, derived on the basis of the following postulated reactions: $E + S \rightleftharpoons ES$ (active); $ES + S \rightleftharpoons ES_2$ (inactive). Analysis of our data for the Mn^{++}-activated arginase in homogenates of mammary gland and
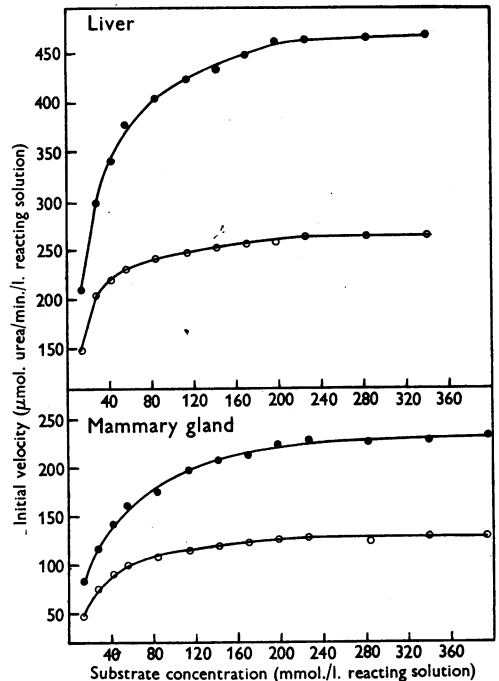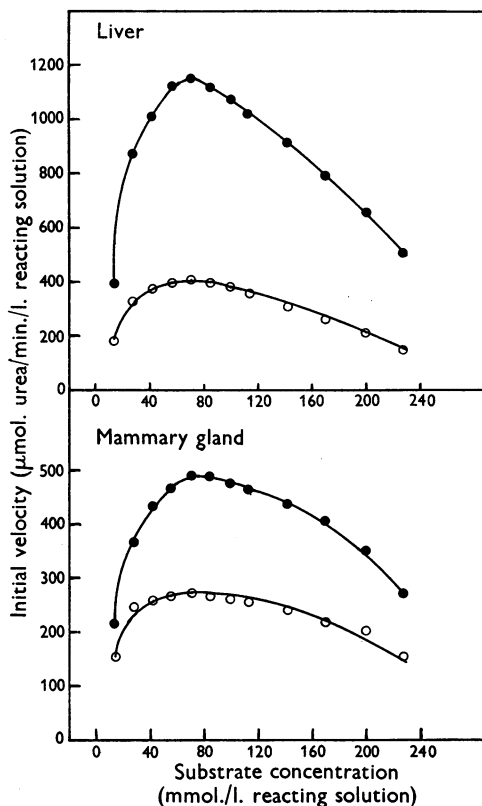

Fig. 9. Relation between substrate concentration and enzyme activity for arginase in homogenates of liver and mammary gland, with (black dots) and without (circles) added Mn^{++}. All experiments at 37° and pH 9·1. Reaction time 10 min.

liver by the methods of Case III of Lineweaver & Burk (1934) gave good agreement (see Fig. 10), over practically the whole range of substrate concentrations studied, with the postulated reactions: $E + S \rightleftharpoons ES$ (active); $ES + 3S \rightleftharpoons ES_4$ (inactive). The values for K_s (or K_m) are given in Table 1, together with those for non-activated homogenates at pH 9·1. Whatever may be the actual mechanism of the substrate inhibition observed with arginase in our experiments, and the above analysis does no more than indicate a possible mechanism consistent with the results, it seems that, at pH values acid to the optimum but not at the optimum itself, arginase

Table 1. *Values of K_m for various arginase preparations*

Enzyme preparation	Determined at		K_m	References
	pH	Temp. (°)		
Rat-liver homogenate + Mn⁺⁺	9·45	37	0·0200	Present investigation
Rat-liver homogenate	9·45	37	0·0100	,,
Rat-liver homogenate + Mn⁺⁺	9·1	37	0·0161	,,
Rat-liver homogenate	9·1	37	0·0177	,,
Rat-mammary-gland homogenate + Mn⁺⁺	9·45	37	0·0200	,,
Rat-mammary-gland homogenate	9·45	37	0·0257	,,
Rat-mammary-gland homogenate + Mn⁺⁺	9·1	37	0·0218	,,
Rat-mammary-gland homogenate	9·1	37	0·0061	,,
Dog-liver extract + Mn⁺⁺	9·5	25	0·0460	Van Slyke & Archibald (1946)*
Purified liver arginase	9·45	40	0·0150	Greenberg & Mohamed (1945)†
Purified liver arginase	9·1	40	0·0130	
Purified ox-liver arginase	9·4	?	0·0118	Damodaran & Narayanan (1940)
Rat-liver slices	7·3	37	0·0025	Bach, Crook & Williamson (1944)
Glycerol extract of rat liver	7·3	37	0·00275	

* Calculated by the method of Lineweaver & Burk (1934) from values read from published curve.
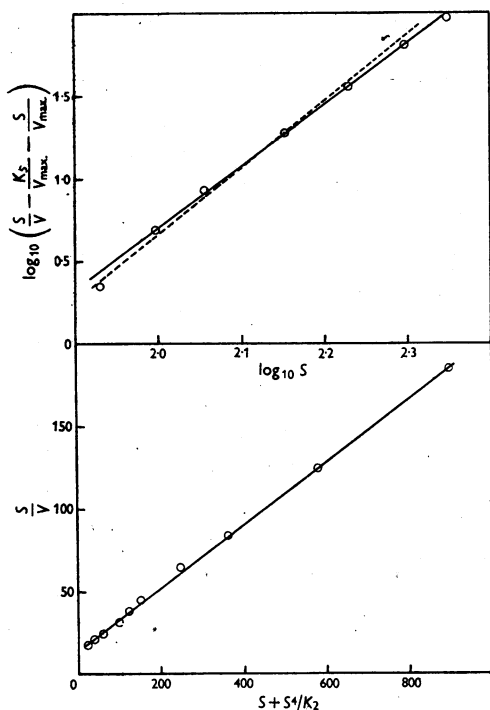† Values read from an apparently conjectural published curve showing relation between K_m and pH.

Fig. 10. Analysis of possible mechanism of inhibition by excessive substrate concentrations of Mn⁺⁺-activated arginase in rat-liver homogenate by the method of Case III of Lineweaver & Burk (1934). On the assumption that inhibition is due to the reactions $E + S \rightarrow ES$ (active); $ES + (n-1) S \rightarrow ES_n$ (inactive), the value of n is given by the slope of the straight line drawn through the experimental points in the upper graph. The dotted line is a line of slope $= 4$. Agreement with theory demands a linear relationship between the functions plotted in the lower graph.

may exist, at least partly, in a form which is able to combine with the substrate in a manner not conducive to hydrolysis.

Michaelis constants (pH 9·45) for activated and non-activated homogenates of liver and mammary gland were evaluated graphically by the method of Case I of Lineweaver & Burk (1934). Plots of S/V against S gave satisfactory linear relationships over the whole range studied (Fig. 11) in agreement with the Michaelis-Menten theory, and confirming the apparent absence at pH 9·45 of inhibition by excessive substrate concentrations. The values for K_m, which, however, were far from constant, are given in Table 1, together with values given by, or calculated from, the results of previous investigators for comparison. The values ascribed to Greenberg & Mohamed (1945) we read from what is evidently a conjectural curve showing K_m as a function of pH; that ascribed to Van Slyke & Archibald (1946) was evaluated by the Lineweaver-Burk method from values we took from their substrate concentration-activity curve. The values quoted from Bach, Crook & Williamson (1944) were determined on rat-liver slices and on a glycerol extract of liver, but their curves are hardly complete enough to justify their use for a simple, direct graphical reading of K_m.

Activation with divalent metal ions

Relative activating effects of Mn⁺⁺ *and* Co⁺⁺. In our studies of the activating effects of Co⁺⁺ (cf. Hellerman & Perkins, 1935; Hunter & Downs, 1944; Mohamed & Greenberg, 1945) relatively small activation effects were observed, rather surprisingly, when homogenates were incubated at 37°, and at a pH and with Co⁺⁺ concentrations found by other workers (e.g. Mohamed & Greenberg, 1945) to suffice for maximal activation.

Experiments in which the degree of activation was measured after various periods of incubation at 37° with Co^{++} showed that, whilst the activity of both liver and mammary-gland homogenates containing no added Co^{++} progressively fell after preparation, even at room temperature, the inactivation was retarded, if not completely prevented, by the presence of Co^{++}. In some experiments the addition of Co^{++} raised the activity slightly above its original value; in others the activity of Co^{++}-treated homogenates showed a progressive fall, but slower and of

Fig. 11. Linear relationship at pH 9·45 between S/V and S for the arginase of rat-liver and mammary-gland homogenates; S =substrate concentration; V =initial reaction velocity.

lesser degree than with untreated homogenates. In one exceptional experiment, addition of Co^{++} produced an unequivocal, though not very striking, increase in activity. A typical experiment is illustrated in Fig. 12. Clearly, under our conditions, Co^{++} does little more than protect the enzyme against progressive inactivation. We thus have a reasonable explanation of the apparent activating effect of Co^{++} on homogenates of mammary gland (e.g. Fig. 1) in contrast to the almost complete lack of effect on liver homogenates, observed in unpublished preliminary experiments with our previous method of determination of arginase activity (Folley & Greenbaum, 1947a). Considerable inactivation of the type just discussed would occur during the 20 hr. hydrolysis at 37° used for mammary-gland homogenates, and if Co^{++} prevented or retarded this process there would apparently be appreciable activation; this

factor would, however, not be so important in the 30 min. hydrolysis used for liver homogenates.

In contrast to the lack of effect with Co^{++}, another activator, Mn^{++} (Klein & Ziese, 1935), was found to exert a striking activating effect on homogenates of liver and mammary gland alike, much of which is so rapid (Fig. 12) as to appear practically instantaneous by the methods used.
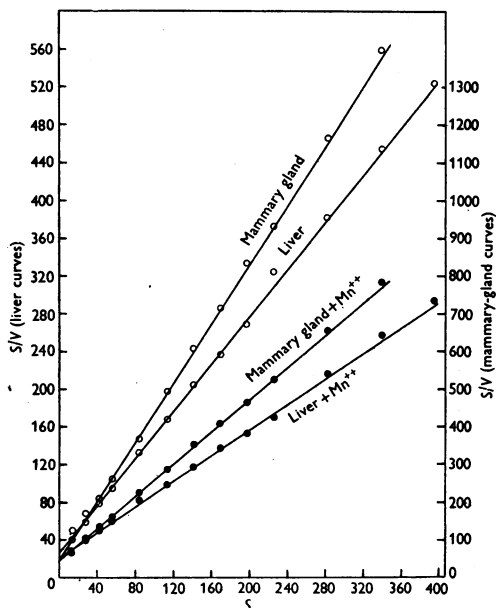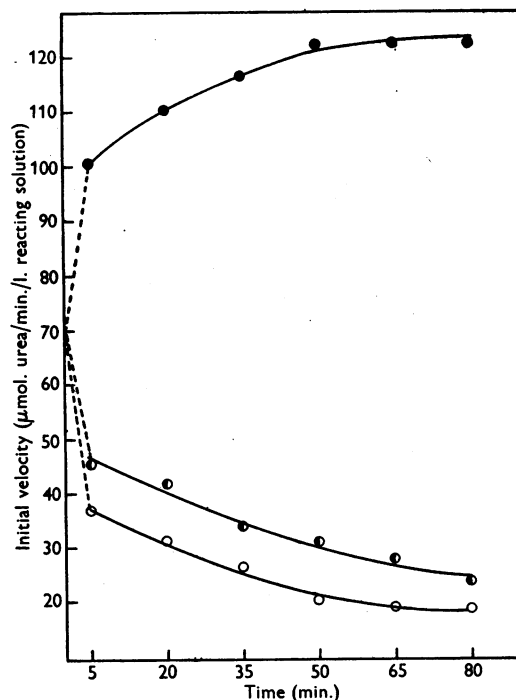

Fig. 12. Effect on the activity of the arginase in a rat-liver homogenate, after dilution 1:200 with saline, of incubation at 37° for various periods (a) in the presence of 2 mg. Mn^{++}/ml. (black dots), (b) 2 mg. Co^{++}/ml. (half dots) and (c) in the absence of added divalent metal ions (circles). All activity determinations were carried out at 37° and pH 9·45; substrate concentration 0·227 M; reaction time 10 min.

In view of the marked and quite unexpected difference thus revealed between the activating effects of Mn^{++} and Co^{++} on the arginase in tissue homogenates, and in order to eliminate the possibility that this might not hold under other conditions, it seemed desirable to investigate the activating effects of Mn^{++} and Co^{++} over a wider range of conditions. Accordingly, factorial experiments (each set up in duplicate) were devised to enable the effect of incubating homogenates for different times with various concentrations of each cation to be studied. In each of two such experiments liver and mammary tissue from a lactating rat were homogenized, and portions of each homogenate at once suitably diluted, each time to the same degree, with saline containing appropriate amounts of $MnSO_4$ or $CoCl_2$. The dilutions were forthwith incubated at 37°, and samples from each taken at appropriate times for enzyme determi-

nation, in one experiment at pH 9·45 and in the other at pH 9·1. It is not possible to show the results graphically, since the set expressing the effect of a given cation on a given homogenate can only be graphically represented by a three-dimensional surface. The results of the experiment in which the enzyme activities were determined at pH 9·45 are, therefore, given in Tables 2 and 3. They define the conditions for maximal activation of the enzyme at homogenate pH and 37° in homogenates of both tissues. All the experiments on arginase activation described in this paper were carried out at homogenate pH, i.e. pH c. 7. Mohamed & Greenberg (1945) found that the rate of activation of their purified arginase by Mn++ increased with rising pH; we

prefer, however, to specify conditions for maximal activation at homogenate pH because of the greater stability of the enzyme protein at neutral reactions.

In confirmation of the previous results in Fig. 12, Tables 2 and 3 show beyond doubt that Mn++ is a much more effective activator of the arginase in our tissue homogenates than Co++, the effect of which is negligible by comparison. These results confirm the previous finding that much of the effect of Mn++ is so rapid as to appear instantaneous with the methods used.

Table 2. *Effect of concentration of* Mn++ *or* Co++ *and of time of incubation at 37° with* Mn++ *or* Co++
on the arginase activity of a rat-liver homogenate

(The values given are initial velocities (μmol. urea/min./l. reacting solution) determined at 37° and pH 9·45; substrate concentration 0·227 M; reaction time 10 min. Values marked * correspond to full activation.)

Time in presence of Mn++ at 37° (min.)	Final concentration of added Mn++ (mg./ml.)						
	0	0·5	1·0	1·5	2·0	2·5	3·0
			Initial velocities (μmol. urea/min./l.)				
0	36·5	79·6	83·7	88·9	94·7	100·5	104·2
15	31·4	90·5	93·2	97·9	103·4	110·0	111·2
30	26·8	96·1	102·0	108·1	111·2	116·3	117·8
45	20·0	102·2	107·8	114·2	117·8	123·3*	124·8*
60	18·9	108·1	111·4	115·8	121·4	124·8*	123·3*
75	18·5	111·2	114·6	123·3*	123·3*	123·3*	124·8*

Time in presence of Co++ at 37° (min.)	Final concentration of added Co++ (mg./ml.)						
	0	0·5	1·0	1·5	2·0	2·5	3·0
			Initial velocities (μmol. urea/min./l.)				
0	36·5	41·1	41·8	43·1	43·9	45·1	45·9
15	31·4	34·6	35·2	40·3	41·1	41·8	41·8
30	26·8	29·8	30·4	32·2	32·8	33·5	34·1
45	20·0	24·8	25·9	29·8	30·3	30·9	30·9
60	18·9	21·1	22·0	27·0	27·6	27·6	27·0
75	18·5	19·4	20·0	23·1	22·6	23·1	23·1

Table 3. *Effect of concentration of* Mn++ *or* Co++ *and of time of incubation at 37° with* Mn++ *or* Co++
on the arginase activity of a rat mammary-gland homogenate

(The values given are initial velocities (μmol. urea/min./l. reacting solution) determined at 37° and pH 9·45; substrate concentration 0·227 M; reaction time 10 min. Values marked * correspond to full activation.)

Time in presence of Mn++ at 37° (min.)	Final concentration of added Mn++ (mg./ml.)						
	0	0·5	1·0	1·5	2·0	2·5	3·0
			Initial velocities (μmol. urea/min./l.)				
0	60·7	136·8	152·7	157·4	164·1	190·8	188·4
15	55·8	144·2	161·0	163·5	181·2	205·5	208·5
30	54·1	153·0	165·1	167·8	192·9	210·0	216·5
45	50·8	158·8	170·6	172·4	206·1	215·0	218·3*
60	51·1	164·0	174·0	177·8	215·4	218·2*	217·5*
75	49·6	169·4	175·8	182·4	219·3*	218·8*	216·5*

Time in presence of Co++ at 37° (min.)	Final concentration of added Co++ (mg./ml.)						
	0	0·5	1·0	1·5	2·0	2·5	3·0
			Initial velocities (μmol. urea/min./l.)				
0	60·7	65·1	66·1	68·0	68·0	68·3	68·0
15	55·8	63·3	64·6	67·0	66·8	66·7	67·0
30	54·1	61·7	63·3	65·1	66·1	66·3	66·1
45	50·8	62·4	64·6	64·8	65·9	67·1	67·2
60	51·1	62·4	65·2	66·1	67·1	67·4	67·2
75	49·6	63·0	65·6	67·1	67·1	67·1	67·6

These findings perhaps indicate that Mn is the actual metal component of the enzyme system in its 'native' state. It seems possible that the 'native' enzyme protein is almost specific in its requirements as regards the metal component, while on purification it becomes increasingly less specific, so that an active arginase can be constituted by addition to the partially purified protein of other divalent metal ions such as Co^{++}, Ni^{++} and Fe^{++}. Thoai, Roche & Roger (1947) have found that alkaline phosphatase, of which Mg^{++} was once regarded as an almost specific activator (e.g. Folley & Kay, 1936), can be reactivated after purification and dialysis by a number of divalent metal ions. In any event, our findings indicate that if homogenates are to be used for studying changes in the fully activated, or what for the present may be termed the 'potential', arginase activity of tissues, it is necessary to use Mn^{++} for the activation process rather than Co^{++} which has been favoured by many investigators of tissue-arginase changes (e.g. Kochakian, 1944). The significance of studies of changes in tissue-arginase activity with Co^{++} added as activator would seem to be somewhat doubtful.

The progressive inactivation of the enzyme in tissue homogenates at pH c. 7 observed at 37° in these experiments, and which also proceeds at an appreciable rate at room temperature, is probably due to the effects of dilution (see also Van Slyke & Archibald, 1946). Dilution of the tissue enzyme systems is an inevitable accompaniment of homogenization, irrespective of any subsequent dilution necessary (e.g. with liver homogenates) to make the enzyme concentration suitable for activity measurements. Such dilution may be assumed to cause gradual dissociation of the metal-protein complex apparently forming the enzyme system (see Hellerman & Stock, 1938). This concept seems to be supported by our finding that this inactivation is reversible, in that not only can the original activity be restored by addition of Mn^{++} to an homogenate which has undergone considerable inactivation due to keeping either at room temperature or at 37°, but also the activity can thus be raised to the higher level characteristic of full activation.

Kinetics of dissociation inactivation and of reactivation by Mn^{++}. The dilution of homogenates during preparation makes it necessary to decide what relationship exists between arginase activities of homogenates, and (a) the actual concentration of the enzyme-protein in the tissues, and (b) the proportion of it which is combined with Mn^{++} thus constituting the active enzyme system (degree of activation). These are clearly questions of considerable importance.

In an attempt to throw light on this problem, the kinetics of dilution inactivation of the arginase in tissue homogenates, and its reactivation by Mn^{++}

were studied by a more refined technique made possible by the increased sensitivity of our new method of arginase activity determination.

Experiments were carried out in which rat liver was homogenized (5 min.) in three portions. One homogenate was, immediately after preparation, diluted 1:8 with normal saline for activity determinations; a second was similarly diluted with saline containing sufficient $MnSO_4$ to give a final concentration of 2 mg. Mn^{++}/ml. In the third case, the liver tissue was homogenized with saline containing sufficient $MnSO_4$ to give the same final concentration of Mn^{++}, and then diluted with the Mn^{++}-saline containing 2 mg. Mn^{++}/ml. All three homogenates were kept at room temperature during the experiment. As soon as possible after dilution, samples of each were taken for arginase determination using 1 min. hydrolysis at pH 9·45, and other samples were similarly taken for enzyme determination, at first at intervals of 0·5 min. and later of 1 min.

Three such experiments were carried out with similar results save in one respect discussed below. One experiment is illustrated in Fig. 13 in which, since each enzyme activity determination occupied 1 min. during which activation or inactivation was proceeding, the first point of each curve was plotted at time 0·5 min. from the instant of dilution instead of at time 0, and the subsequent points plotted accordingly. The same plotting procedure was adopted for Fig. 12.
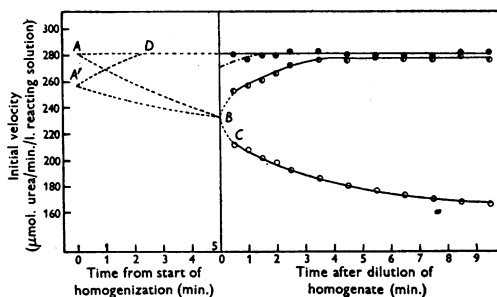

Fig. 13. Changes with time in the activity of the arginase present in homogenates of a rat liver. One homogenate was diluted with normal saline immediately after preparation (circles); another was similarly diluted with saline containing Mn^{++} (half dots). In the third case Mn^{++} was present during homogenization and Mn^{++}-saline used for the dilution (black dots). After dilution the three preparations were kept at room temperature and samples taken at intervals for activity determination. Enzyme activities were determined at 37° and pH 9·45; substrate concentration 0·227M; reaction time 1 min. For explanation of dotted portion of curves, see text.

The curves for the two homogenates, in which no added Mn^{++} was present during homogenization, have been extrapolated back to intersect at zero time, i.e. the instant at which the dilution was performed. In making this conjectural extrapolation, it is assumed that the activities of the two homogenates changed at approximately the same rate, so that the curves are made to intersect at a point about midway

between their initial values. The point of inter-
section (B) represents the activity of the homogenate
just prior to dilution. This, however, is probably
somewhat less than the activity of the enzyme as it
existed in the tissue, since some dissociation of the
metal-enzyme must have occurred during homo-
genization. It is evident that dilution of a freshly
prepared homogenate causes considerable loss of
activity even at room temperature; in the experi-
ment illustrated in Fig. 13 there was an approxi-
mately 15 % drop over the first 5 min. in the activity
of the homogenate diluted with normal saline.

The activity of the preparation homogenized with
Mn^{++} remained constant at a value which was
approximately equal to the 'ceiling' value attained
by the homogenate activated with Mn^{++}-saline after
preparation. Furthermore, the activity of the homo-
genate containing no Mn^{++} could be raised to the
same level when Mn^{++} was added at the end of the
experiment, thus confirming our previous finding
that dilution inactivation is reversible.

These results seem to be susceptible of two inter-
pretations. On the one hand it may be assumed that
the enzyme was fully activated in the intact tissue,
and that the activity of the preparation homogenized
with Mn^{++} represents, as nearly as can be deter-
mined by present methods, the true tissue arginase
activity. The fall in activity which occurs during
homogenization in the absence of added Mn^{++} can
then be reasonably represented by some such curve
as AB in Fig. 13. On the other hand it is equally
possible that in the tissue the enzyme was not com-
pletely activated, and that in the preparation homo-
genized with Mn^{++} activation of the enzyme up to
the full 'ceiling' value occurred during this process.
In this case the 'native' activity may be represented
by some point A' intermediate between A and B
(Fig. 13), and the course of activation during the
homogenization in the presence of Mn^{++} by $A'D$.
At present there is no obvious way of deciding be-
tween these alternatives, either of which may hold
for the particular liver in question. The results of
the other two experiments seemed to indicate that
the arginase in the two livers concerned could not
have been fully activated in the 'native' state, since
in each case the Mn^{++}-homogenized preparation
showed some rise in activity at the beginning of the
series of determinations (depicted by the part-
dotted line in Fig. 13), which doubtless represented
the concluding stages of an activation process
initiated during homogenization. It is hardly
possible to speculate in greater detail about the
course of events during homogenization since, in the
present state of knowledge, these are bound to be
obscure; but it seems safe to conclude from these
experiments that the degree of activation of the
arginase system *in vivo* cannot be determined by
present methods. More work will be necessary before

it will be possible to evaluate the prospects of deter-
mining the true arginase activity of a given tissue.

In the meantime, determinations of the 'potential'
tissue arginase, i.e. of the level of enzyme protein
in the tissues, a measure of which is given by the
fully activated arginase activity of an homogenate
(Mn^{++} not Co^{++} must be used), would seem to be
of greater biological significance than determinations
of arginase carried out in absence of Mn^{++}, though
such values (e.g. those of Folley & Greenbaum,
1947 a, b), provided they are made under strictly
standard conditions as regards dilution and are per-
formed as soon as possible after homogenization of
the tissues, are almost certainly capable of leading
to valid conclusions regarding relatively large
changes in tissue arginase.

Reliance on determinations of the fully activated
arginase, though this represents the best that can be
done at present, is, however, not entirely satis-
factory, since we are faced with the difficulty of
deciding whether observed changes in tissue arginase
activity are due to changes in the concentration of
the active Mn^{++}-protein or merely to alterations in
the proportion of an unchanged amount of protein
which is combined with Mn^{++}. At present there
seems to be no satisfactory way of deciding between
these alternatives.

SUMMARY

1. The arginase present in homogenates of rat
liver and mammary gland has been studied by
means of a sensitive reaction-velocity method in-
volving colorimetric determination of urea.

2. The reaction velocity at the pH optimum and
in presence of excess substrate (0·227 M) is sensibly
linear over the first 10 min., so that, provided not too
much substrate is hydrolyzed, amounts of urea
formed in periods up to 10 min. are proportional to
initial velocities, and can be converted into suitable
arginase units without use of an enzyme calibration
curve. Generally similar results were obtained at
pH 9·1 in presence of 0·071 M-substrate.

3. The optimal pH (9·45) in presence of 0·227 M-
substrate was the same for homogenates of both
tissues, and was unaffected by full activation with
Mn^{++}; the optimal pH was hardly affected by
decreasing the substrate concentration to 0·071 M.

4. At the optimal pH there was no evidence of
enzyme inhibition by excessive substrate concen-
trations, the results agreeing well with the Michaelis-
Menten theory, but at pH 9·1 such inhibition
occurred and progressively increased as the sub-
strate concentration was increased above the
optimum (0·071 M).

5. In the absence of added Mn^{++}, the arginase
activity of liver and mammary-gland homogenates
progressively falls even at room temperature and
pH 7. The rate of inactivation is greater the more

dilute the homogenate, and it is doubtless due to gradual dissociation of the metal-protein complex forming the enzyme. The inactivation can be reversed by addition of Mn^{++} (full activation taking an appreciable time even at 37°), or prevented if Mn^{++} is present from the outset. Co^{++} does not reactivate partially inactivated homogenates very much, and if present from the outset does little more than prevent or retard dilution inactivation. Mn^{++} and not Co^{++} is the metal component of the arginase system in the tissues, the enzyme system being reversibly dissociable.

6. Studies of the kinetics of dissociation inactivation and reactivation by Mn^{++} indicate that the enzyme may not always exist in the fully activated state *in vivo*. There is no known method by which the degree of activation of tissue arginase can be determined, and most biological significance attaches to the concentration of enzyme protein (i.e. potential arginase) in the tissues, a measure of which is given by the activity of a homogenate fully activated by Mn^{++}.

7. The enzymes in liver and mammary-gland homogenates are so similar in properties that they can be considered identical. In some respects the enzyme in tissue homogenates probably behaves more like the 'native' enzyme than does that in partially purified preparations. Homogenates are thus preferable to extracts for tissue arginase studies.

8. A routine method for the determination of tissue arginase is described. It involves homogenization of the tissue, and the determination of the arginase activity of the homogenate, fully activated by Mn^{++}, with only 5 min., and sometimes only 1 min., hydrolysis.

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