The chief object of this paper is to present certain improvements in Hunter and Dauphinee's method (1) for the measurement of arginase activity. Incidentally we describe a procedure for the concentration and partial purification of arginase, and report some observations upon its activation by cobalt.

In principle the method of Hunter and Dauphinee consisted in determining the amount of urea produced by a known volume of the arginase-containing solution acting, for a given time and under certain defined conditions, upon a known quantity of arginine, and referring this to a standard curve showing the empirically ascertained relation between urea production (under the chosen conditions) and quantity of enzyme. The same principle had been used already by Edlbacher and Röthler (2).

Continued use of the method has revealed certain defects in the original prescription. (a) No care was taken to provide that, whether in the preparation of the reference curve or in the testing of an unknown, the volume of the enzyme-substrate mixture (and therefore the concentration of the arginine) should be always the same. (b) The action of the enzyme was measured in a phosphate mixture at a pH (8.4) at which the buffering effect of phosphate is very slight. (c) The unit of arginase activity was defined from a point of the reference curve so near the origin that its exact location was largely guesswork. (d) The crude jack bean extract, employed as a source of urease, contained itself, as subsequently discovered by Hellerman and Perkins (3, 4), enough arginase to introduce an appreciable error into the determination.

Revising the method in the light of these considerations we have (a) adopted for all arginine-buffer-arginase mixtures a uniform volume of 8 ml., with a final arginine concentration of 0.02225 M, (b) substituted for phosphate a phosphate-phenolsulfonate mixture of the same pH and the same total buffer concentration, (c) redefined our arginase unit, and (d) replaced the excess of crude jack bean extract originally employed by an adequate but not unnecessarily large amount of a partially purified urease. In addition we have thought it advisable (e) to purify to some extent the arginase used in the construction of the standard curve and (f) to activate it fully with cobalt.
Buffer Solution—The buffer solution now used has the following composition: 0.5 M sodium p-phenolsulfonate 50 ml., M KH$_2$PO$_4$ 25 ml., 2 N NaOH 15.6 ml., water to 100 ml. This solution is 0.5 M in total buffer concentration, and has a pH of 8.4.

Unit of Arginase Activity—The unit of arginase activity we now define as one-tenth of that amount of arginase, which, under standard conditions (as defined later), liberates urea equivalent to 2.5 mg. of nitrogen. Upon the curve relating enzyme quantity to action (Fig. 1) the accurate location of the 2.5 mg. point presents no difficulty. On the original curve of Hunter and Dauphinee (1) this point, as it happens, corresponds to exactly 10 units of arginase. We have satisfied ourselves that the change in composition of the buffer is without appreciable effect upon the activity of the enzyme. The new and more precise method of defining the unit leaves its magnitude, therefore, unaltered.

Preparation of Suitable Urease Solution—The preparation of a urease absolutely devoid of argininosylytic properties appears to be a difficult matter, but the following procedure will usually give a product suitable enough for the purpose in view. Jack bean meal, 100 gm., is extracted with 500 ml. of water, and the centrifuged extract is treated with 8 volumes of acetone. The precipitate is collected by centrifugation, dried over H$_2$SO$_4$, and ground to a fine powder. This is again extracted, this time with 400 ml. of water, and the processes of precipitation, drying, and grinding are repeated, to yield finally 20 to 25 gm. of powder. For use 0.5 gm. of this powder is dissolved in 100 ml. of 60 per cent glycerol. 1 ml. of this solution should decompose in 1 hour at room temperature urea equivalent to 9 to 10 mg. of nitrogen. Coincidental action upon arginine should be negligible. To test the latter point we determine the amount of ammonia nitrogen liberated during 1 hour at room temperature by the action of 1 ml. of the urease solution upon a mixture of 2 ml. of buffer solution, 5 ml. of 3 per cent arginine hydrochloride solution, and 1 ml. of 0.04 per cent cobalt nitrate. Although this mixture contains 4 times the standard amount of arginine, the yield of ammonia nitrogen ought not to exceed 0.02 mg. By way of contrast it may be reported that 1 ml. of a crude urease solution, prepared according to the directions of Schmidt (5), has been found to yield under similar conditions as much as 0.4 mg.

Preparation of Partly Purified Arginase Concentrate—The enzyme solution used in preparing the original standard curve of reference was a suitably diluted crude liver extract. We have replaced this by a solution containing a smaller proportion of inert material, and, with other purposes in view, we find it convenient to combine partial purification with concentration. A fresh baby beef liver is drained as completely as possible of blood, and minced. 1 kilo of the moist product is stirred for 10 minutes with 1 liter
of water. The mixture is transferred to a flask, submerged in a bath of water at 65°, and stirred gently until the mass reaches a uniform temperature of 58°. After being maintained at this temperature for 5 minutes, the mixture is rapidly cooled and centrifuged. A measured volume, say 800 ml., of the supernatant is treated with 1.2 volumes of acetone. The resulting flocculent precipitate is separated as quickly as possible by centrifugation, and drained of adherent liquid. It is then taken up in 400 ml. (0.5 volume) of water, and stirred or shaken until as much as possible has gone into solution. Undissolved material is removed by centrifugation, and the clear supernatant is mixed with 1.2 times its volume of acetone. Again the mixture is rapidly centrifuged, the second acetone precipitate is treated like the first, and its clarified solution is subjected to a third precipitation. The third precipitate is centrifuged off and transferred to a vacuum desiccator. The desiccator is evacuated step by step, until all the acetone and most of the water have been evaporated. The residual waxy paste is then ground up thoroughly with 75 per cent glycerol in one-tenth of the initial volume (80 ml.). The turbid reddish brown solution thus obtained will contain at least half of the arginase originally present, so that the concentration of the enzyme will have been increased 5 times or more. Different preparations have contained, after full activation by cobalt, from 500 to 900 or more units per ml. The degree of purification attainable may be exemplified by one instance, in which the number of arginase units per mg. of N rose from 21 in the crude liver extract up to 73 in the final product.

A concentrate prepared in the way described and preserved at 10° will retain its original activity almost unimpaired for a year. Diluted with water it forms a milky suspension from which there gradually separates a more or less copious flocculent precipitate of water-insoluble protein. This precipitate carries down with it a part of the enzyme, so that the clear supernatant is only 85 to 90 per cent as active as the whole suspension. On the other hand the supernatant has a slightly higher ratio of arginase to nitrogen.

Activation of Arginase Solutions—The activity of arginase solutions or suspensions prepared from liver is increased by treatment with certain metals, such as manganese (6) or cobalt (3, 4, 7). This activation, like that of intestinal peptidase by manganese (8), is a time reaction. This is shown by such experiments as the two recorded in Table I.

In Experiment 1, 0.3 ml. of an arginase concentrate was mixed with 14 ml. of water at 37°. The dilute suspension was divided into two equal portions, A and B, each of which was thenceforward kept at 37°. To Portion B was added 0.05 ml. of a 2 per cent solution of anhydrous cobaltous nitrate. As soon thereafter as possible the first activity determina-
MEASUREMENT OF ARGINASE ACTIVITY

tions were started with 1 ml. portions, first of Portion A and then of Portion B. Other determinations were commenced 10, 20, 60, and 180 minutes later. The technique of these determinations was that prescribed later, and involved an action period of 30 minutes.

Experiment 2 differed in that the original diluted suspension (not the same as that of Experiment 1) was centrifuged, and only the supernatant solution was treated with cobalt. Such treatment, it may be noted, leads to the gradual formation, in the originally clear mixture, of a fairly bulky precipitate. A part of the total active enzyme is adsorbed on, or otherwise incorporated in, this precipitate. The activities recorded in the present experiments are those of the whole mixture, precipitate included.

TABLE I

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Nature of arginase preparation</th>
<th>Time at 37° before activity determination</th>
<th>Portion A, without cobalt</th>
<th>Portion B, with cobalt</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>min.</td>
<td>mg.</td>
<td>units per ml.</td>
</tr>
<tr>
<td>1 Suspensions</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Suspension</td>
<td>3</td>
<td>2.033</td>
<td>7.1</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>15</td>
<td>2.033</td>
<td>7.1</td>
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<tr>
<td>1</td>
<td></td>
<td>63</td>
<td>1.976</td>
<td>6.8</td>
</tr>
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<td>1</td>
<td></td>
<td>183</td>
<td>1.866</td>
<td>6.2</td>
</tr>
<tr>
<td>2 Supernatant</td>
<td></td>
<td></td>
<td>2.063</td>
<td>7.3</td>
</tr>
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<td>2</td>
<td></td>
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<tr>
<td>2</td>
<td></td>
<td>63</td>
<td>1.967</td>
<td>6.8</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>183</td>
<td>1.902</td>
<td>6.4</td>
</tr>
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</table>

The time intervals, as given in Table I, are reckoned from the instant at which cobalt was added to Portion B of the enzyme solution. They define only the moments of sampling. The measured activities cannot be taken as the actual activities at these precise moments, for it can hardly be doubted that activation (or inactivation), unless already maximal, progressed appreciably during the 30 minutes required for each determination. This inherent ambiguity does not affect the general conclusions to be drawn from the data, (1) that in the absence of cobalt the diluted enzyme is gradually destroyed, and (2) that in the presence of the metal its activity progressively increases. Independent observations of similar import, but with manganese as activator, have been made by Archibald at the Hospital of the Rockefeller Institute. Dr. Archibald found that at 50° maximal activation is attained in as little as 20 minutes. This we have since ascertained to be true for cobalt activation also. Evidence to that effect will

1 Archibald, R. M., private communication.
be found in Table II, which incorporates the results of various experiments in which different dilutions of one arginase concentrate (the same as that of Table I) were exposed to the action of cobalt for various times at 20°, 37°, or 50°. In these experiments (the first with Preparation 4 excepted) the concentration of cobalt was 2.5 times greater than in the previous ones. From the results it appears that at 20° maximal activation requires more than 48 hours, at 37° more than 3 but not more than 24 hours, and at 50° not more than 20 minutes. It is also shown that the activated enzyme, in

<table>
<thead>
<tr>
<th>Temperature °C.</th>
<th>Diluted arginase preparation</th>
<th>Time with cobalt at given temperature</th>
<th>Activity</th>
<th>Increase of activity</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Preparation No.</td>
<td>Nature</td>
<td>hrs.</td>
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<tr>
<td>20</td>
<td>3</td>
<td>Supernatant</td>
<td>24</td>
<td>6.8</td>
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<tr>
<td></td>
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<td>48</td>
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<td>&quot;&quot;</td>
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</tr>
<tr>
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<td>Suspension</td>
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<tr>
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<td>3</td>
<td>7.3</td>
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<tr>
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<td>Supernatant</td>
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<tr>
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<td></td>
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<td>&quot;&quot;</td>
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<td>7.35</td>
</tr>
<tr>
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<td>Suspension</td>
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<tr>
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<td>20</td>
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<td></td>
<td>7</td>
<td>&quot;&quot;</td>
<td>40</td>
<td>6.75</td>
</tr>
</tbody>
</table>

* Followed by 24 hours at 37°.

contrast with the unactivated, is very stable, withstanding without loss exposure to 50° for 20 minutes more or to 37° for 24 hours.

We have mentioned already that, when a clear centrifuged solution of arginase is treated with cobalt, a part of the enzyme is precipitated in active form. At 50° this part amounts, we find, to 20 to 30 per cent of the total activated enzyme. Thus, for example, 5 ml. portions of the three cobalt-treated mixtures which appear last in Table II (with total activities of 15.0, 15.0, and 14.9 units per ml.) were centrifuged, and the precipitate in each case (not wholly free from adherent liquid) was redispersed in 5 ml. of 0.05 per cent cobalt nitrate. The activities of the supernatants were then found to be respectively 11.1, 11.15, and 11.0 units per ml., those
of the precipitates 4.6, 4.05, 4.3 units per ml. It will be seen that the whole activity was recovered in the sum of the two fractions, and that, in this instance, the proportion remaining in solution was 74 per cent. In other instances it has been as high as 80 and as low as 71 per cent.

Preparation of Diluted and Activated Arginase Solution—For the construction of the standard reference curve one requires an arginase solution containing from 16 to 20 units of enzyme per ml.; that is to say, a solution, of which 1 ml., acting under standard conditions (as defined later) upon a standard amount of arginine, will liberate between 3.25 and 3.5 mg. of urea nitrogen. Such a solution is obtained by appropriate dilution of any glycerol concentrate. If the potency of the latter and its behavior upon activation are unknown, the dilution required, which may vary between 15- and 40-fold, will have to be ascertained by trial. The process of dilution is conveniently combined with that of activation. For the latter we have adopted the conditions recommended by Archibald. The procedure is accordingly as follows:

1 ml. of the concentrate is mixed with the required amount of water. A 5 per cent solution of anhydrous cobalt nitrate is added at the rate of 0.06 ml. for each 10 ml. of the mixture. This is enough to insure maximum activation and an optimum concentration of cobalt in the final digest. The mixture is now set in a water bath at 50° and left there for 20 minutes. The flocculent precipitate, which separates, is centrifuged off, and only the clear, practically colorless supernatant is made use of. This may be only 60 to 70 per cent as active as the uncentrifuged mixture, for the centrifuge removes not only the originally suspended arginase (10 to 15 per cent of the whole) but also that precipitated by cobalt (20 to 30 per cent of the remainder). The homogeneity of the product is an obvious counterbalancing advantage. It may be added that centrifugation removes not only active enzyme but also some inert nitrogenous material, for in the final supernatant the ratio of arginase to nitrogen is greater than in the original concentrate. In one instance it rose from 52 to 64 units per mg. of nitrogen, and in another it even reached 100.

Construction of Standard Reference Curve

Reagents—

Dilute arginase solution, as described in the preceding section.

Arginine hydrochloride, 1.875 per cent solution in the 0.5 M phosphate-phenolsulfonate buffer mixture of pH 8.4 already described. 2 ml. of this solution contain 9.975 mg. of nitrogen and will yield therefore a maximum of almost 5 mg. of urea nitrogen.

Urease solution, 0.5 per cent in 60 per cent glycerol. The preparation of, and specifications for, this solution have been given earlier.
Hydrochloric acid and sodium hydroxide solutions, each about 2 N.
Phenol red indicator solution, 0.05 per cent.
Standard acid, standard alkali, and indicator as required for the micro-
titrimetric determination of ammonia.

Procedure—Into each of ten Van Slyke-Cullen urea tubes are measured 
2 ml. of the buffered arginine solution. To these tubes, numbered con-
secutively, there are further added 5.9, 5.8, . . . 5.1, and 5.0 ml. of water.
Into an ordinary test-tube are put a few ml. of the arginase solution. All 
the tubes are then set in a thermostat at 37°.

When the tubes have reached the temperature of the bath, 0.1 ml. of 
the arginase solution is transferred to Tube 1, the contents are rapidly 
mixed, and the time is noted. To Tubes 2 to 10 there are added in the 
same way at exactly measured convenient intervals 0.2, 0.3, . . . 0.9, and 
1.0 ml. respectively of arginase. It will be remarked that the total volume 
of each completed mixture is the same, namely 8 ml. The concentration 
of arginine in each is 0.02225 M.

To each tube in turn, about 29 minutes after the addition of the arginase, 
there is added a drop of phenol red indicator. Exactly upon the 30th 
minute the tube is removed from the thermostat, and treated with as many 
drops of 2 N HCl as are required to turn the indicator bright yellow.
The action of the arginase having been thus inhibited, the contents of the 
tube are boiled, until the protein present has been completely coagulated.

The tubes having been cooled, each is treated drop by drop with 2 N 
NaOH, sufficient to give a just perceptibly pink color (pH about 6.8).
To each is added finally 1 ml. of the urease solution. This is allowed to 
act at room temperature for 1 hour, and the urea determinations are com-
pleted in the usual way. The results are to be corrected by a control, in 
which a mixture of 2 ml. of arginine-buffer solution and 6 ml. of water, 
adjusted to a pH of about 6.8, is treated with urease in the same way as the 
principal mixtures.

A graphical representation of the results enables one to estimate the 
volume of arginase solution, which would give exactly 2.5 mg. of urea 
nitrogen. This volume contains, by definition, 10 units of the enzyme. 
On this basis the ml. of arginase solution are translated into units of 
arginase. The final reference curve is plotted accordingly, with mg. of 
urea nitrogen as ordinates and arginase units as abscissae. The curve 
thus obtained is reproduced in Fig. 1. In spite of the many extra precau-
tions taken in its preparation, it differs but little from the original curve of 
Hunter and Dauphinee.

In the interest of accuracy it is best to restrict the use of the curve to 
the part between 1.25 and 3 mg. of nitrogen (3.5 to 14 units) of arginase. 
With higher yields of nitrogen the flattening out of the curve increases the 
significance in arginase units of any given error in the urea determination.
With lower yields the same absolute error becomes an unduly large fraction of the true value.

Measurement of Arginase Activity—The reagents required are those listed in the preceding section, with the exception, of course, of the dilute arginase solution. The place of the latter is taken by the solution of which the activity is to be determined. This will usually have to be diluted. For fresh liver extracts a 10-fold dilution will generally be about right; for concentrates a 20-fold or even a 100-fold dilution may be necessary. The correct proportion can be ascertained only by trial.

The procedure is as follows: Into one urea tube are measured 2 ml. of the arginine-buffer solution; into another, serving as a control, 2 ml. of plain buffer solution. To each there are added 5 ml. of water. Both tubes are then set in the thermostat at 37°, along with a test-tube containing some of the solution of which the arginase activity is to be tested.

As soon as it is certain that the tubes have attained the temperature of the bath, 1 ml. of the enzyme solution is transferred (a) to the arginine tube and (b) to the control. The time of each addition is noted. From this point both tubes are treated in the manner described in the preceding section, so that the contents of each are acidified and boiled exactly 30 minutes after the addition of the enzyme, then neutralised, and submitted to a urea determination. The main result, corrected by the control, should fall between 1.25 and 3 mg. of urea nitrogen. If it is outside these

![Fig. 1. Standard reference curve for measurement of arginase activity](http://www.jbc.org)
limits the process should be repeated with a more appropriate dilution of
the original enzyme solution. When an acceptable result is obtained, it
is referred to the standard curve, from which one may read directly the
number of arginase units in the 1 ml. of diluted enzyme solution used.

SUMMARY

Details are given of an improved method for the measurement of arginase
activity.

A procedure is described for the concentration and partial purification
of liver arginase.

The activation of arginase by cobalt is shown to be a time reaction.

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THE MEASUREMENT OF ARGINASE ACTIVITY

Andrew Hunter and Clarence E. Downs


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