A Short Method for the Purification of Arginase from Ox Liver

By S. J. BACH, R. A. HAWKINS AND D. SWAINE

Department of Physiology, University of Bristol

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The preparation of highly purified arginase solutions (Bach & Killip, 1958, 1961) proved somewhat laborious. Further work led to a much shorter and easily reproducible method by the use of which an extract of arginase from acetone-dried powder of ox liver could be purified to the precrystallization stage in 2 days of laboratory work.

MATERIALS

L(+)-Arginine hydrochloride was obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A. The Arlco jack-bean meal used in the preparation of urease was supplied by Hopkin and Williams Ltd., Chadwell Heath, Essex.

The 'manganese-maleate solution' used in the preparation of arginase was made by dissolving 8.9 g. of $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ and 1.8 g. of disodium maleate in glass-distilled water and making the volume up to 1 l.

Buffers. 0.1M-Glycine buffer was prepared by dissolving 7.5 g. of glycine in 200 ml. of water, adjusting the pH of the solution to 9.0 with 0.1N-NaOH and making the volume up to 1 l. with glass-distilled water. 3M-Acetate buffer, pH 4.6, was prepared by dissolving 408 g. of hydrated sodium acetate in 500 ml. of water, adding 170 ml. of acetic acid and making the volume up to 1 l. with glass-distilled water.

METHODS

Definition and determination of arginase activity. The enzyme activity was defined and determined as described by Bach & Killip (1958), as also was the method of determining protein.

Preparation of urease. Jack-bean meal was treated with acetone as described by Sumner (1955). The acetone mixture was filtered and the precipitate, obtained from the filtrate after standing for 24 hr., was taken up in 40 ml. of glass-distilled water to yield the urease stock solution which was free from canavanine. For the use of urease in the manometric method of urea determination, 1 ml. of 3M-acetate buffer, pH 4.6, was added to 2 ml. of urease stock solution immediately before the experiment.

Preparation of acetone-dried liver powder. Fresh ox liver (1 kg.), freed from connective tissue, was cut into five or six pieces which were rinsed under the tap and minced. Portions of mince (200 g.) were homogenized in a Waring Blender with 200 ml. of cold iso-osmotic KCl solution for 20 sec. and the homogenate was mixed immediately in the blender with 200 ml. of acetone at -10° for another 20 sec. The acetone-treated homogenate was then poured with stirring into a 10 l. beaker containing 2.5 l. of acetone at

-10° . When the final portion of mince had been treated, the contents of the beaker were made up with cold acetone to a volume of 10 l. and mixed. The mixture was held at 4° for 2 hr. The clear supernatant was decanted and the content of the beaker made up again with acetone to 10 l. After a further 2 hr. at 4° the clear supernatant was decanted and the suspension rapidly filtered on a Buchner funnel which was covered with a rubber sheet to exclude as much air as possible. Before the cake on the funnel was completely dry, it was washed with 2 l. of cold acetone; then the filtration was continued until the particles were completely dry. The solid material, packed into a hard pad, was broken up, spread out on filter paper and air-dried. The powder was finally ground in a mortar and stored *in vacuo* at 4° ; it retained its activity for several months. The yield was 250 g. of powder.

RESULTS

Purification of arginase from acetone-dried powder of ox liver

Step 1. Extraction. A portion (100 g.) of acetone-dried powder was stirred slowly with 1 l. of tap water contained in a 5 l. dark-glass bottle. After a few minutes, 5.9 g. of manganese sulphate tetrahydrate was added to the mixture, the pH of which was adjusted to 6.8 with N-sodium hydroxide. Then a further 600 ml. of tap water was added, followed by a further 5.9 g. of manganese sulphate tetrahydrate and by a readjustment of the pH. A final portion of 400 ml. of tap water was added and the stirring continued until 80 min. after the beginning of the extraction. The mixture was filtered through glass wool and the pH of the filtrate adjusted to 7.3. The precipitate that formed in the filtrate was separated by centrifuging from the dull-orange supernatant fluid (solution A).

Step 2. Fractionation with acetone. To 400 ml. of solution A at $1-4^\circ$, contained in a 2 l. conical flask, 240 ml. of acetone at -10° was slowly added. The content of the flask was swirled in a freezing mixture of solid carbon dioxide and acetone. The temperature of the reaction mixture was not allowed to exceed $4-6^\circ$ during the acetone treatment. A precipitate formed, which contained the enzyme, and was separated by centrifuging. The residual supernatant fluid was drained off by inverting the centrifuge vessels; most of the precipitate dissolved in approx. 100 ml. of manganese-maleate solution.

This suspension was kept cold during the treatment of the remainder of solution A. The total volume of manganese-maleate solution used in this step was 450 ml. The bulky precipitate formed in the suspension on standing was separated by centrifuging and discarded; the dark-red supernatant solution containing the enzyme was dialysed for 20 hr. against 10 vol. of manganese-maleate solution which was renewed once during the dialysis. The non-diffusible material, freed from a small precipitate formed during the dialysis and with its pH readjusted to 7.3, yielded a dark-red solution C.

Step 3. Heat treatment. Solution C was heated in a 2 l. flask to 30° and then immersed in a water bath at 65–70° with vigorous swirling until the temperature of the solution had risen to 55°. This temperature was maintained for 3 min., after which the flask was rapidly cooled and the bulky buff precipitate discarded by centrifuging. The dark-red supernatant solution D contained the enzyme.

Step 4. Ammonium sulphate fractionation. Solution D was 50% saturated with ammonium sulphate and the inactive precipitate was separated by centrifuging. The pale-yellow supernatant solution (F₁) was further treated with ammonium sulphate to attain a saturation of 65%. The enzymic precipitate was taken up in 40 ml. of manganese-maleate solution to yield a yellow-green solution F₂. In this step the bulk of the red pigments was removed.

Step 5. Treatment with propan-2-ol. Solution F₂ was cooled to 1° and then slowly treated with 1.1 vol. of propan-2-ol at –10°. The bulky enzymic precipitate was centrifuged off at 3000g and dissolved in 40 ml. of manganese-maleate solution. The

mixture, which contained some insoluble material, was dialysed overnight against 10 vol. of manganese-maleate solution, after which the insoluble material was separated from the slightly-yellow supernatant fluid G₁ which contained the enzyme.

Step 6. Second ammonium sulphate fractionation. Solution G₁ was saturated to 47% with ammonium sulphate. The substantial precipitate was centrifuged down at 3000g and discarded. The colourless supernatant solution was allowed to stand for 48 hr., during which time a further precipitate was formed. This was discarded by centrifuging and the supernatant solution G₂ was further treated with ammonium sulphate to attain a saturation of 58%. The enzymic precipitate formed was taken up in about 15 ml. of freshly prepared manganese-maleate solution. The specific activity of this solution (G₃) should be at least 100 000.

The course of the purification of the enzyme is outlined in Table 1.

Stability of arginase in the various stages of purification

The preparations after steps 1 and 2 retained 75% of their original activities after 2 weeks at 4°. The solutions obtained in steps 4 and 5 were slightly more stable than those obtained from steps 1 and 2. At stages D and G₃ the preparations retained their activities over a period of 2 months when stored at 4°. In the final G₃ stage the enzyme solution can be stored at –15° for an indefinite period. The G₃ solution can also be freeze-dried to yield a white powder which, on dissolving in manganese-maleate solution, was found to have retained 65% of its original specific activity.

Table 1. *Course of purification of arginase from acetone-dried ox liver*

Step	Fraction	Solution	Vol. (ml.)	Activity (units/ml.)	Protein N (mg./ml.)	Specific activity (units/mg. of protein N)	10 ⁻⁶ × Total units	Yield (%)
1	Supernatant obtained from extract after centrifuging	A	1670	17100	3.05	5900*	28.6	100
2	Fraction precipitated by 37.5% (v/v) acetone, after dialysis	C	660	31500	2.92	9400	20.8	78
3	Supernatant obtained after heating at 55° and centrifuging	D	568	29900	1.3	23000	17.0	60
4a	Supernatant obtained at 50% ammonium sulphate saturation	F ₁	597	19450	0.71	27400	11.6	41
4b	Fraction precipitated by ammonium sulphate (50–65% saturation)	F ₂	68	154000	3.66	42000	10.5	37
5	Fraction precipitated by 52.5% (v/v) propan-2-ol after dialysis	G ₁	78	88000	1.13	78000	6.9	24
6a	Supernatant obtained at 47% ammonium sulphate saturation after standing for 48 hr.	G ₂	78	71000	0.71	100000	5.5	19
6b	Fraction precipitated by ammonium sulphate (47–58% saturation)	G ₃	17	226500	1.7	133000	3.8	13.5

* The specific activity at step A represents a 25-fold purification over that of the enzyme in crude liver homogenate.

DISCUSSION

The comparatively laborious procedure of purification of arginase, as described by Bach & Killip (1958, 1961), has been considerably simplified, so that the enzyme can now be extracted from acetone-dried ox-liver powder and purified to the precrystallization stage in less than 2 days of laboratory work. By elaborating the mode of the ammonium sulphate fractionation, the method has become easily reproducible and may lend itself to teaching experiments. From the final stage of the purification procedure, the enzyme can be crystallized in one further step, by using the procedure reported by Bach & Killip (1958).

SUMMARY

1. A comparatively short method for the preparation of arginase from ox liver is described which permits the purification of the enzyme from acetone-dried liver powder to the precrystallization stage in 2 days of laboratory work.

REFERENCES

- Bach, S. J. & Killip, J. D. (1958). *Biochim. biophys. Acta*, **29**, 273.
Bach, S. J. & Killip, J. D. (1961). *Biochim. biophys. Acta*, **47**, 336.
Sumner, J. B. (1955). In *Methods of Enzymology*, vol. 2, p. 378. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

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The Distribution of Organically Bound Sulphate in Bone and Cartilage during Calcification

By J. A. WEATHERELL AND S. M. WEIDMANN

Biological Research Unit, Leeds University School of Dentistry, Leeds 1

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Wherever calcification takes place, sulphated mucopolysaccharides seem to be present. They are found in sites of osteogenesis (Kent, Jowsey, Steddon, Oliver & Vaughan, 1956), are a principal constituent of calcifying cartilage (Dziewiatkowski, Di Ferrante, Bronner & Okinaka, 1957), and occur during dentine formation (Léblond, Bélanger & Greulich, 1955). As a result of this, sulphated mucopolysaccharides have for many years been linked with the processes of hard-tissue formation.

Despite the considerable coincidence between the occurrence of these sulphate esters and the deposition of mineral salts, their role in the process of calcification is still not sufficiently understood. There is disagreement whether these substances assist calcification, or prevent it. Some workers have postulated that their presence is associated with the initiation of calcification (Rubin & Howard, 1950; Sobel, Burger & Nobel, 1960), whereas others (Logan, 1935; Sylvén, 1947; Glimcher, 1959) suggested that their removal promotes mineralization of the tissue.

The changes that occur in the concentration of organically bound sulphate in a tissue during its mineralization have not been clearly established, and the present work was undertaken to obtain information about the concentrations of these substances in different regions of calcifying epiphyseal cartilage of ox foetus.

MATERIALS AND METHODS

Epiphyseal cartilage from both ends of the femora of foetal ox was selected for examination. The fetuses chosen were approx. 45 cm. in length, nose to anus, taken from mixed breeds of cattle at the Leeds City Abattoir. The cartilaginous epiphyses were removed from both ends of the femora, and either dissected and analysed immediately or deep-frozen until use. The epiphyses contained no secondary centres of ossification and their large size permitted a number of analyses to be carried out in a continuous series from the non-calcifying cartilage of the hyaline region to the primary bone spicules of the metaphysis.

Distribution of samples. After the epiphyseal cartilage was removed from the femora, the outer surface of articular cartilage was cut away and the remaining material sliced sagittally into sections 0.5 mm. thick. From these sections, narrow strips of cartilage parallel to the growth plate were cut under the dissecting microscope and pooled into samples representing six regions of cartilage as shown in Fig. 1. The first sample, *A*, represented the uncalcified hyaline region of the epiphyseal head. Hyaline cartilage was usually separated from the growth plate by a narrow band of optically dense tissue, *B*, rather similar in appearance to hyaline cartilage but less basophilic and perhaps more cellular. This area, which will be called the 'dense' region, constituted the second sample. The third, fourth and fifth samples, *C*, *D* and *E* respectively, were adjacent areas of the epiphyseal plate, the third being next to the dense area, and the fifth lying against the translucent region of hypertrophied and degenerate chondrocytes. The sixth area, *F*,