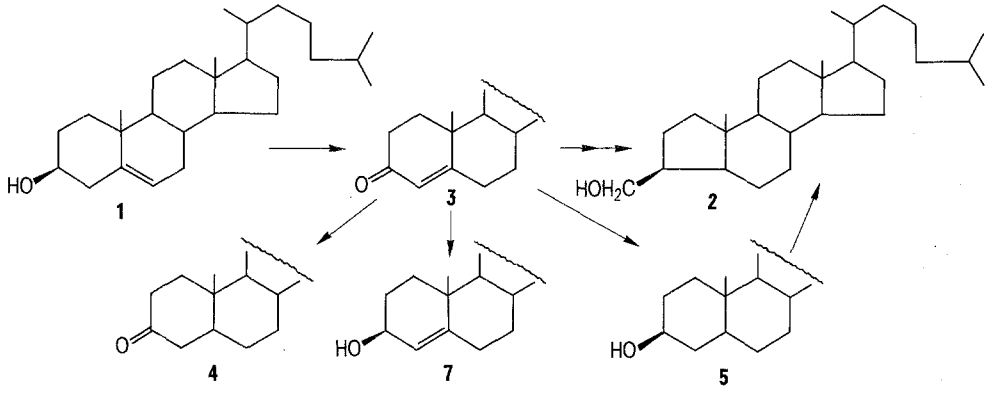
The failure to detect any radioactivity in the 3β-formyl-A-nor-steranes (part structure **6**) fraction (experiment 4, table 2) is convincing evidence that this aldehyde is not a product of the metabolism of cholesterol in the sponge, whereas different amounts of radioactivity were found associated with 5α-cholestan-3-one (**4**) and cholest-4-en-3β-ol (**7**) indicating that cholest-4-en-3-one (**3**) is reduced to these latter in the sponge.

In order to evaluate the role of these reduction products as intermediates in the conversion cholesterol/3β-hydroxymethyl-A-nor-cholestane 2 separate cold trap experiments were performed (experiments 5 and 6). The results (table 2), showing that low radioactivity is associated with the supposed intermediates, indicate that 5α-cholestan-3-one (**4**) and cholest-4-en-3β-ol (**7**) represent side reaction products of the metabolic fate of cholest-4-en-3-one (**3**). The results presented in this paper are summarized in the scheme.



Scheme. Intermediates and side products in the conversion of cholesterol into 3β-hydroxymethyl-A-nor-cholestane.

1 M. De Rosa, L. Minale and G. Sodano, Experientia *31*, 408 (1975).
2 L. Minale and G. Sodano, J. chem. Soc. Perkin I *1974*, 2380.
3 M. De Rosa, L. Minale and G. Sodano, Experientia *32*, 1112 (1976).
4 L. Bibolino, L. Minale and G. Sodano, J. chem. Soc. chem. Commun. *1978*, 524.
5 R.V. Oppenauer, Org. Synth. coll. Vol. *3*, 207 (1955).
6 A.G. Smith and L.J. Goad, Biochem. J. *146*, 35 (1975).
7 J.S. Pizey, in: Synthetic Reagents, vol.2, p.143. Ellis Horwood Ltd, Chichester 1974.
8 R. Evans and J.R. Hanson, J. chem. Soc. Perkin I *1975*, 663.
9 J.E. Graebe, P. Hedden and J. MacMillan, J. chem. Soc. chem. Commun. *1975*, 161.
10 M. Node, H. Hori and E. Fujita, J. chem. Soc. Perkin I *1976*, 2144.

Purification of canine myocardial mitochondrial creatine kinase[1]

R. Roberts[2]

Cardiovascular Division, Washington University School of Medicine, 660 South Euclid Avenue, St. Louis (Missouri 63110, USA), 3 September 1979

Summary. Mitochondrial creatine kinase, purified for the first time, is a dimeric molecule with a mol.wt of 82,000 and does not hybridize with M or B subunits or react to their specific antiserum. A specific antiserum to the mitochondrial form was developed which does not cross-react with the B or M subunits. Thus, the mitochondrial form is biochemically and immunologically unique.

Creatine kinase (EC 2.7.3.2) is known to exist in 3 molecular forms (isoenzymes) which based on their subunit composition are referred to as BB, MB and MM creatine kinase. These isoenzymes are present in the cytosol and based on their different net charge in an alkaline medium can be separated by electrophoresis. Mitochondria[3] are known to be rich in creatine kinase which on electrophoresis is positively charged exhibiting a distinctly different electrophoretic mobility from that of the cytosolic isoenzymes. However, skepticism remains since only 2 subunits are known. Attempts to purify mitochondrial creatine kinase have been unsuccessful due in part to its chemical lability and consistent contamination with MM creatine kinase[4,5]. In the present report, mitochondrial creatine kinase has been obtained in pure form and shown to be immunologically and biochemically distinct from that of cytosolic creatine kinase isoenzymes.

Material and methods. Creatine kinase activity was determined by the coupled enzyme system as previously described[6] and isoenzymes analyzed by electrophoresis[7]. Sodium-dodecyl-sulphate polyacrylamide gels were performed according to the method of Fairbanks et al.[8] and molecular weight assessed by comparison with markers of known molecular weight according to the method of Weber and Osborn[9]. Creatine kinase isoenzymes MM and MB were purified from dog heart and brain as previously described[10] and labelled with ^{125}I[11]. Mitochondrial CK was labelled with ^{125}I and specific antiserum developed according to the methods used for the development of antisera for cytosolic CK isoenzymes[12,13].

Canine hearts for purification of mitochondrial creatine kinase were removed immediately after sacrifice of the animal and minced with scissors and homogenized in a Potter-Elvehjem power homogenizer. Homogenizing medium (10 ml/g) contains sucrose (0.075 M), mannitol (0.225 M), EDTA (0.002 M) and MOPS (0.05 M) at a pH of 7.2. After homogenization, centrifugation was performed at 1000 × g (4 °C) for 10 min. The supernatant was centrifuged

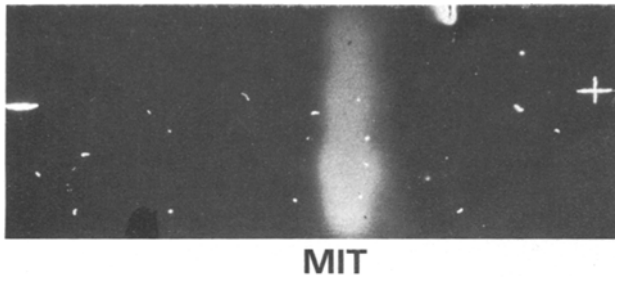


Fig. 1. Electrophoresis of the final preparation on cellulose acetate showed a single mitochondrial (MIT) isoenzyme band which is positively charged and migrating to the negative (−) electrode. Cytosolic creatine kinase (MB and BB) migrate towards the positive electrode (+) and MM remains at the origin (white dot at the top).

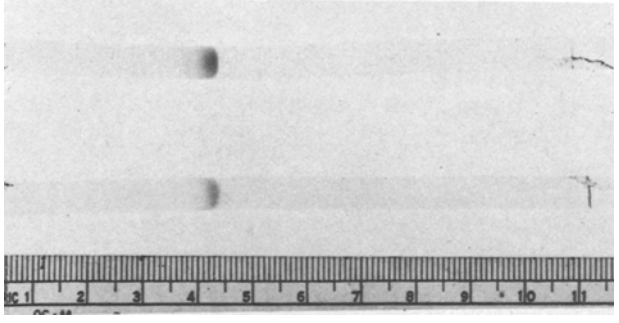


Fig. 2. Following electrophoresis on sodium-dodecyl-sulphate-polyacrylamide gel, the mitochondrial preparation exhibited a single protein band as shown in the gel on the right. For comparison is shown the results of MM creatine kinase on your left. The position occupied by the mitochondrial creatine kinase is virtually identical to that occupied by MM creatine kinase.

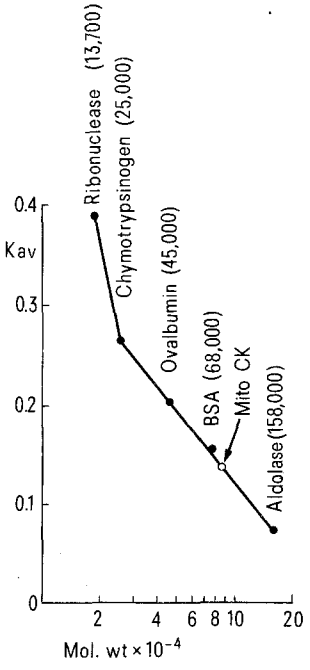


Fig. 3. Molecular weight of the intact mitochondrial creatine kinase (mito CK) was determined on a G150 sephadex column. Comparison of mitochondrial creatine kinase with markers of known molecular weight indicate a mol.wt of about 82,000.

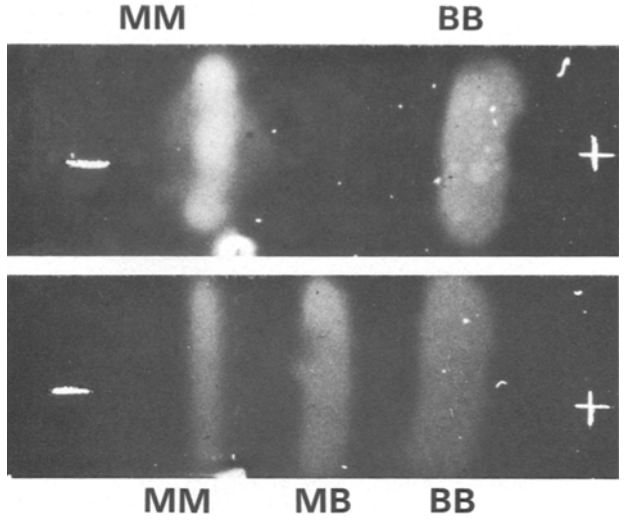


Fig. 4. Shown here are photographs of the results after electrophoresis before and after repeated freezing and thawing of solutions of MM and BB creatine kinase. The top panel shows the 2 isoenzymes clearly separated prior to freezing and thawing. The bottom panel showing the formation of the hybrid from MB creatine kinase is after freezing and thawing.

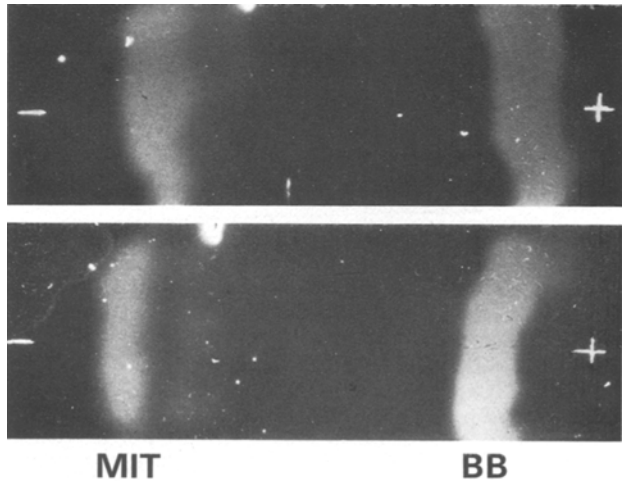


Fig. 5. Shown here are photographs of the actual results after electrophoresis before and after repeated freezing and thawing of solutions containing mitochondrial and BB creatine kinase. Results in the top panel (before) are identical to those in the lower panel (after). Note the absence of any hybrid form.

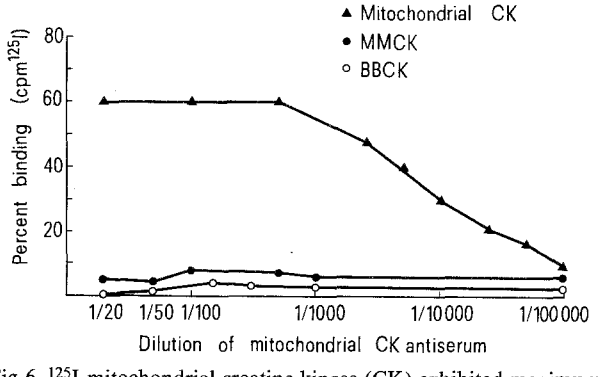


Fig. 6. ^{125}I-mitochondrial creatine kinase (CK) exhibited maximum binding (60%) at a titre of 1:1000 but only 7% binding at a titre of 1:100,000. Mitochondrial creatine kinase antiserum exhibited no binding to ^{125}I-MM or ^{125}I-BB creatine kinase. Thus, the antiserum is specific for mitochondrial creatine kinase.

at 15,000 × g for 10 min at 4 °C and this process repeated times 3. The pellet was recovered and incubated in a total volume of about 8 ml of Na_2HPO_4 (0.083 M), pH 7.5 for 20 min at 26 °C to release CK from the mitochondria. Following centrifugation (50,000 × g) the supernatant was recovered and applied to sephacryl S-200 column (1.5 × 90 cm, Pharmacia) with a bed volume of 159 ml and a flow rate of 30–34 ml/h. Elution was performed with Tris barbital (0.050 M, pH 7.8) containing mercaptoethanol (0.005 M) in fractions of 2 ml. The fractions containing activity were further fractionated on CM sephadex cation exchange column using a Glenco column (2.6 × 30 cm) with a bed volume of about 40 ml and a flow rate of 1 ml/min. Elution was performed with Tris barbital (0.050 M), pH 7.8) containing mercaptoethanol (0.005 M) in fractions of 2 ml. 2 peaks of activity were observed with the initial peak being MM and the 2nd peak mitochondrial creatine kinase.

Results and discussion. The final fraction from CM-sephadex was shown by electrophoresis on cellulose acetate to exhibit a single band which was positively charged and free of MM creatine kinase (figure 1). On SDS-polyacrylamide gels, a single protein band was observed with a mol.wt of about 44,000 (figure 2). On a G-200 sephadex column, the mol.wt was estimated to be 84,000 (figure 3), thus mitochondrial creatine kinase is a dimer composed of 2 monomers of identical molecular weight. Repeated freezing and thawing (× 6) of solutions of MM and BB creatine kinase consistently exhibited the hybrid form MB (figure 4), however silimar experiments performed between mitochondrial creatine kinase and that of BB or MM consistently exhibited no hybrid form (figure 5).

Antiserum developed to mitochondrial creatine kinase exhibited binding to ^{125}I-mitochondrial creatine kinase which was dependent on the antibody concentration but exhibited no binding to MM or BB creatine kinase (figure 6). Antiserum to MM and BB creatine kinase exhibited no binding to ^{125}I-mitochondrial creatine kinase (figure 7).

Thus, in conclusion mitochondrial creatine kinase does not hybridize with B or M subunits and exhibits biochemical and immunological properties not shared by that of the B or M subunits. Whether it is synthesized in the mitochondria by its own DNA or transported following synthesis in the nucleus is unknown.

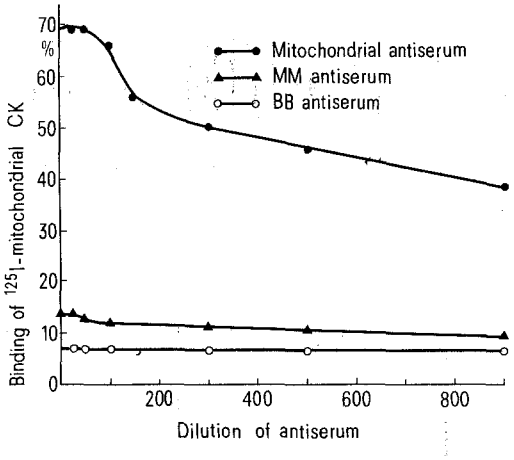


Fig. 7. Shown here is further evidence for the specificity for mitochondrial antiserum. The upper curve shows the binding curve between ^{125}I-mitochondrial creatine kinase and that of mitochondrial antiserum. However, antiserum to MM or BB exhibited no binding to mitochondrial creatine kinase indicating complete lack of cross-reactivity.

1 Supported in part by the Special Center of Research in Ischemic Heart Disease (1 P17 HL 17646).
2 I thank Ann Grace for her technical assistance and Ava Ysaguirre for typing the mansucript.
3 W.E. Jacobus and A.L. Lehninger, J. biol. Chem. *248*, 4803 (1973).
4 E.C. Farrell, N. Baba, G.P. Brierley and H.C. Grumer, Lab. Invest. *27*, 209 (1972).
5 N. Hall, P. Addis and M. DeLuca, Biochem. biophys. Res. Commun. *76*, 950 (1977).
6 R. Roberts, P.D. Henry and B.E. Sobel, Circulation *52*, 743 (1975).
7 R. Roberts, K.S. Gowda, P.A. Ludbrook and B.E. Sobel, Am. J. Cardiol. *36*. 433 (1975).
8 G. Fairbanks, T.L. Steck and D.F.H. Wallach, Biochemistry *10*, 13 (1971).
9 K. Weber and M. Osborn, J. biol. Chem. *242*, 4406 (1969).
10 E. Carlson, R. Roberts and B.E. Sobel, J. molec. Cell Cardiol. *8*, 259 (1976).
11 A.E. Bolton and W.M. Hunter, Biochem. J. *133*, 529 (1973).
12 R. Roberts, B.E. Sobel and C.W. Parker, Science *194*, 855 (1976).
13 R. Roberts and A. Painter, Biochim. biophys. Acta *480*, 521 (1977).

Effect of fluoride on in vivo nitrate reduction in rice leaves (*Oryza sativa* L.)

Srinivasan and C.S. Rama Rao[1]

Division of Biochemistry, Indian Agricultural Research Institute, New Delhi-110012 (India), 20 September 1979

Summary. Fluoride had no effect on in vitro nitrate reductase activity in rice leaves, but in vivo activity was strongly inhibited. It is suggested that fluoride brings about this inhibition by adversely affecting the physiological NADH generating system required for in vivo nitrate reduction.

Fluoride has long been known to be injurious to plants. Large scale damage to vegetation in some areas by industrial waste has been shown to be due to accumulation of fluoride[2]. Effects of fluoride on plants are diverse. Visible symptoms of fluoride injury like retarded growth, necrosis and chlorosis of tissues are preceeded by pronounced biochemical changes. The effects of fluoride on protein and nucleic acid metabolism[3,4], respiration[5–7] and photosynthesis[8,9] have been reported. At the enzyme level, a number of enzymes including ATPase[10], hexokinase[11], enolase[12], phosphoglucomutase[13], succinic and malic dehydrogenases[14,15] are inhibited by fluoride, while other enzymes such as glucose-6-phosphate dehydrogenase, catalase, peroxidase and cytochrome oxidase are stimulated[16]. Very little information is, however, available on the effect of fluoride on nitrate assimilation in plants. Nitrate reductase, an enzyme which catalyses the first step in nitrate assimilation is considered to be the rate limiting step in the