pooled and saved for use as a background blank in ultraviolet absorption spectrophotometry and mass spectrometry. 1,25-$(OH)_2D_3$-like activity was monitored on all columns by the radioreceptor assay of Brumbaugh *et al.* (*4*).

18. Ultraviolet absorption studies were performed on a dual-beam Beckman DB spectrophotometer fitted with a recorder. Because of the minimal absorbance present in the sample, a small amount of background absorbance from the Celite column or solvents, or both, was subtracted by reading the peak fractions off the last 1 by 40 cm Celite column (fractions 20 to 27) in 1 ml of distilled ethanol against an equal number of fractions obtained just prior to and after the peak (fractions 16 to 19 and 28 to 31), also in 1 ml of distilled ethanol.
19. A model 3200F-6103 Finnigan GC/MS system with quadrapole analyzer was utilized for mass spectrometry. Samples were directly introduced on the probe and continuous scanning was carried out while the samples were rapidly heated to 275°C above ambient temperature; ionization was accomplished by electron bombardment. Preliminary analysis was done on 500 ng of 1,25-$(OH)_2D_3$ and the synthetic hormone emerged from the probe at 225°C and produced the characteristic mass spectrum of this molecule, as published by Holick *et al* (*3*). One microgram of purified hydrolyzed *S. malacoxylon* was similarly analyzed, as was a comparable portion of the background blank saved from the final Celite column.
20. A 2-meter 3 percent QF1 (50 percent trifluoropropylmethyl silicone, Alltech, Ill.) column was employed for gas chromatography. The column was heated to 200°C above ambient temperature.
21. The differences noted in the mass spectral patterns between Fig. 1A and Fig. 1B suggest the formation of cyclized pyroderivatives during the gas chromatographic phase. Vitamin D and 1,25-$(OH)_2D_3$ are known to undergo a thermal cyclization reaction, yielding pyrocalciferols [H. Ziffer, W. J. A. Van den Heuvell, E. O. A. Haahti, E. C. Horning, *J. Am. Chem. Soc.* **82**, 6411 (1960); J. W. Blunt, H. F. DeLuca, H. K. Schnoes, *Biochemistry* **7**, 3317 (1968)]. The transformation apparently takes place in the "flash heating" zone of the gas chromatography column and, in the present case, produces 1$\alpha$,25-dihydroxypyrocholecalciferol. Evidence for this cyclization is the striking diminution of the fragment at *m/e* 134, which in direct probe analysis is the major species and represents the ring A moiety plus C-6 and C-7 of the open triene system. Moreover, fragments at *m/e* 209 (*m/e* 251 minus 42) [H. Budzikiewicz, C. Djerassi, D. H. Williams, *Structure Elucidation of Natural Products by Mass Spectroscopy* (Holden-Day, San Francisco, 1964), vol. 2, p. 94] and 155, 141, 105, 69, and 55 are consistent with various cleavages of the fused ABCD ring array of classic steroids [G. R. Waller, *Biochemical Applications of Mass Spectroscopy* (Wiley-Interscience, New York, 1972), p. 278].
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## Radioimmunoassay for Creatine Kinase Isoenzymes

Abstract. *Creatine kinase has three isoenzymes designated MM, MB, and BB, with BB being the brain form and MM the muscle form. Antibodies to BB creatine kinase were obtained by immunization of rabbits with human BB creatine kinase. The antibodies demonstrated specificity for BB and MB creatine kinase (myocardial isoenzyme), but no cross-reactivity with MM creatine kinase. With the use of this antibody, a highly sensitive radioimmunoassay capable of measuring picomolar amounts of MB creatine kinase has been developed. Clinical application of this method should provide a sensitive and specific test for the diagnosis of myocardial infarction.*

Creatine kinase (E.C. 2.7.3.2) is a dimeric molecule that exists in at least three isoenzyme combinations [each with a mass of approximately 82,000 daltons (*1*)] designated MM, MB, and BB on the basis of monomer composition, M being the predominant form in muscle and B being the predominant form in brain. Plasma from normal human subjects contains primarily MM, with less than 0.005 international unit (IU) of MB per milliliter and no detectable BB creatine kinase. The only human tissue containing appreciable amounts of MB creatine kinase is myocardium (*2*), and elevated MB creatine kinase activity in plasma is a remarkably sensitive and specific marker indicator of myocardial injury. Accordingly, analysis of serial changes in plasma MB creatine kinase activity has been utilized to estimate the extent of acute myocardial infarction in experimental animals and patients (*3, 4*). Results of such estimates are directly dependent on several parameters, one of which is the rate of disappearance of MB creatine kinase from plasma. Unfortunately, factors responsible for the disappearance of MB activity from the circulation in particular, and disappearance of activity of numerous other enzymes used as markers of organ injury in general, have not been well elucidated (*4*). Furthermore, it is not clear whether the disappearance of enzyme activity is rate-limited by inactivation, denaturation, or removal of intact enzyme molecules from the circulation. Despite the general similarity of individual creatine kinase isoenzymes, the rates of disappearance of their activities from the circulation are strikingly different and are not directly related to their lability in solution in vitro (*5*).

Ischemic heart disease is the most common cause of death in the Western world and in the United States alone it accounts for over 750,000 deaths per annum. The prevalence of myocardial infarction is high but the diagnosis is often difficult to confirm. Accordingly, the need for improved diagnostic tests with increased sensitivity and specificity exists. The present study was undertaken to develop a sensitive and specific assay for quantitative detection of isoenzymes of CK containing the B subunit based on physical properties of the enzyme molecules as well as isoenzyme activity. Analysis of profiles of creatine kinase isoenzymes in human tissue indicates that brain contains only BB (*2, 6*), skeletal muscle only MM (*2, 6, 7*), and heart, a combination of MM and MB (*2, 6*). Nevertheless, after brain injury, BB does not appear in the circulation, as shown by studies of patients with cerebral infarction, injury, or infection (*8, 9*). This may be due to the blood brain barrier, or lability of BB creatine kinase in cerebral spinal fluid, or blood itself, although the reason for the lack of appearance of BB has not been established. After cerebral damage, plasma MM is often elevated, presumably because it is released from skeletal muscle as a result of sympathetic stimulation (*10*). Analysis of creatine kinase isoenzyme activity in the plasma after intramuscular injections (*11*) and surgical procedures (*2*) demonstrates that plasma MM creatine kinase may be markedly elevated as a result of these procedures. However, neither BB nor MM increases in concentration. Since BB creatine kinase does not appear in human plasma after myocardial infarction (*7*), the availability of such an assay for the B subunit should be useful in characterizing the rate and nature of disappearance of MB creatine kinase isoenzyme activity from the circulation and in facilitating detection and quantification of myocardial infarction. Although several assays for MB creatine kinase activity have been developed (*6, 11, 12*), they are not ideally suited for quantitative analysis of large numbers of samples: their sensitivity is somewhat limited, and they rely exclusively on detection of enzyme activity rather than other physical properties of creatine kinase isoenzyme molecules.

MM and MB isoenzymes were prepared from human myocardium (obtained at necropsy within 3 hours of death) and BB was prepared from human brain as recently described (*13*). In brief, these tissues were homogenized in 0.05*M* tris-HCl (*p*H 7.4) containing 0.001*M* 2-mercaptoethanol, centrifuged at 31,000*g*, and extracted repetitively with ethanol. Individual isoenzymes were precipitated with 70 percent ethanol, resuspended, separated completely from each other by batch adsorption and column chromatography with diethylaminoethyl cellulose-

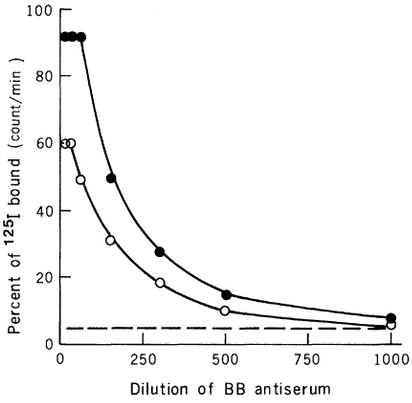


Fig. 1. The specificity of BB antiserum for $^{125}$I-labeled BB (solid circles) and $^{125}$I-labeled MB (open circles). Dashed line is for MM creatine kinase. The binding of BB and MB depends on the concentration of the BB antibody. There is no binding of $^{125}$I-labeled MM creatine kinase at any concentration of BB antibody.

Sephadex A50, dialyzed, freeze-dried, and stored at 0° to 4°C.

Antibodies to human creatine kinase isoenzymes were obtained by immunizing rabbits with human MM and human BB creatine kinase mixed with equal volumes of Freund's complete adjuvant. Initially, the rabbits were injected subcutaneously with 1 mg of immunogen (0.25 mg per foot pad). Subsequently, they were injected with 0.25 mg weekly for 3 weeks. All animals were given booster injections of 0.1 mg in complete adjuvant at monthly intervals thereafter. Ten days after each booster injection, the animals were bled and their serum was analyzed for antibody activity. Ouchterlony agarose plates, prepared with the BB antiserum, exhibited a single precipitin line to BB and MB antigens but no precipitin line with MM. Plates prepared with MM antiserum exhibited a single precipitin line to both MB and MM but none with BB. These results suggested that the antibodies harvested were specific for the B or M subunits rather than the isoenzyme molecules as a whole.

Radioactive iodine was used to label creatine kinase isoenzymes for subsequent use in a competitive displacement radioimmunoassay. When $^{125}$I was introduced into the isozymes by the chloramine-T or lactoperoxidase methods there were marked losses of creatine kinase activity, possibly because of oxidation of essential sulfhydryl groups. To avoid exposing the enzymes to oxidizing agents and contaminants in the radioactive iodine, the $^{125}$I was first incorporated into *N*-succinimidyl 3-(4-hydroxyphenyl-proprionate) by the method of Bolton and Hunter (*14*). The $^{125}$I-labeled *N*-succinimidyl 3-(4-hydroxphenyl-proprionate) was in turn reacted with amino groups of the protein. After purification the labeled ester was combined with 2 to 8 mg of MM, MB, or BB creatine kinase in 1 to 2 ml of 0.01*M* sodium borate buffer, *p*H 8.5. The reaction mixture was shaken for 15 minutes at 4°C, and then the labeled isoenzymes were dialyzed against the same buffer containing 0.002*M* 2-mercaptoethanol. Radioactivity per microgram of labeled creatine kinase isoenzyme averaged 46,000 count/min for MM and MB creatine kinase, and 10,000 count/min for BB creatine kinase. The maximum loss of enzyme activity resulting from labeling and dialysis was less than 5 percent for each isoenzyme preparation.

The binding activity of the BB antibodies for $^{125}$I-labeled BB, MB, and MM was studied at high antibody concentrations (dilutions of 1:5 to 1:30). Antibody-bound creatine kinase was separated from the creatine kinase that remained free by ammonium sulfate precipitation (*15*). Greater than 50 percent binding was obtained with the BB and MB markers (maxium binding levels were 93 and 60 percent, respectively, compared with only 3 percent binding of the MM marker) (Fig. 1).

The converse result was obtained with the MM antiserum, with 93 and 56 percent binding of the MM and MB markers, respectively, and only 2.6 percent binding of the BB marker. With all three markers, normal rabbit serum produced only 2 to 3 percent binding. Thus, the binding of the $^{125}$I-labeled antigens was immunologically specific and there was no apparent cross-reactivity between the MM and BB systems.

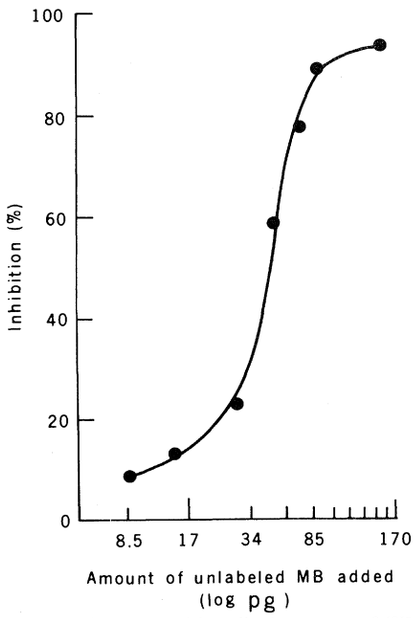


Fig. 2. The competitive displacement of $^{125}$I-labeled MB creatine kinase by unlabeled MB creatine kinase, a reaction that is concentration-dependent. The inhibition curve is steep, in the range of 17 to 80 pg.

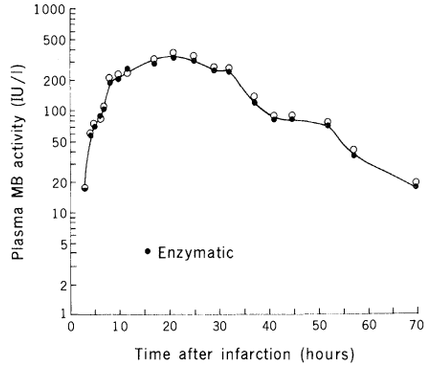


Fig. 3. The activity of creatine kinase in plasma from a patient with myocardial infarction plotted against time. Since 1 microgram of purified MB enzyme protein was equivalent to 0.017 IU of enzyme activity, values obtained with the radioimmunoassay method expressed initially as nanograms per milliliter, were converted to enzymatic activity as shown with the open circles. Results were compared to those obtained by a kinetic fluorometric assay which measures enzymatic activity, shown with solid circles. As can be seen, values obtained with the two methods are in close agreement. Similar agreement was obtained in all 60 samples from the three patients studied.

The specificity of the BB antiserum for B subunits was further evaluated in a competitive displacement binding radioimmunoassay comparing the ability of unlabeled BB, MB, and MM creatine kinase to inhibit the binding of $^{125}$I-labeled BB creatine kinase. BB and MB produced a dose-related diminution of binding of $^{125}$I-labeled BB, with 50 percent inhibition occurring at 26 and 51 pg, respectively, and essentially complete inhibition of binding occurring at concentrations of 170 pg/ml and above (Fig. 2). With MM creatine kinase, no inhibition of binding of $^{125}$I-labeled BB occurred even at 4.2 $\mu$g/ml (a 25,000-fold molar excess over $^{125}$I-labeled BB). Moreover, the inhibition curve with unlabeled MB was unaltered in the presence of high concentrations of MM. Thus in the BB system, the competitive displacement assay for MB not only is very sensitive but can detect small quantities of MB in the presence of much larger quantities of MM.

In patients with acute myocardial infarction, the peak MB creatine kinase activity in plasma generally represents 10 to 15 percent of peak total creatine kinase activity (*6*). Thus, the molar ratio of MM to MB is much less than the 25,000-fold ratio utilized above in evaluating the specificity of the B subunit radioimmunoassay. Furthermore, while re-

finements to distinguish BB from MB may eventually be desirable, BB activity is generally not present in plasma from patients with acute myocardial infarction and accordingly displacement binding will, under almost all circumstances, reflect MB exclusively. These impressions are corroborated by parallel radioimmunoassay and enzyme measurements in serial determinations performed in 60 samples from three patients with infarction; the results from one patient are illustrated in Fig. 3.

The high level of specificity in the radioimmunoassay system for creatine kinase isoenzyme suggests that a similar approach may be useful in differentiation of other clinically important enzymes which exist in multiple forms. Previous assays for plasma enzymes and isoenzymes have been based on enzymatic activity. Studies evaluating the disappearance of the enzymes from the circulation have been restricted to determining the loss of activity. However, since this assay detects the concentration of molecules, one can determine the actual rate of isoenzyme turnover independent of activity. The assay should help to elucidate mechanisms responsible for disappearance of individual creatine kinase isoenzymes from the circulation as well as aid in elucidating the relative importance of inactivation, denaturation, or removal of creatine kinase molecules under various clinical circumstances.

Even more important, because of its sensitivity and potential for detecting enzymatically inactive MB creatine kinase isoenzymes in the circulation, the creatine kinase radioimmunoassay may lead to improved enzymatic estimates of infarct size as well as earlier detection of acute myocardial infarction. The mean activity of MB creatine kinase in the plasma of normal subjects is only 0.002 IU/ml. Thus, even a two- to threefold increase would be difficult to detect reliably with conventional methods whereas the present method detects 0.0001 IU/ml. In view of the recent evidence that ischemic myocardium can be protected in patients with acute infarction, it is important that a definitive diagnosis be made as soon as possible, since agents that can potentially decrease infarct size would be more effective if they were administered soon after the development of the initial symptoms.

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## Early Eye Removal Produces Excessive Bilateral Branching in the Rat: Application of Cobalt Filling Method

Abstract. *When one eye of a rat is removed at birth, axons from the remaining eye form an excess of branches which are directed to both sides of the brain. This finding, which is based on a novel application of cobalt tracing methods, provides an explanation for previous reports of expanded uncrossed projections after early eye removal.*

The formation of organized pathways in the central nervous system must include both the directed growth of an appropriate number of axons, sometimes over long distances, and the spatial ordering of their synaptic connections within target nuclei. Considerable attention has been devoted to the problem of the formation of connections in the brain, but relatively little is known about the factors which direct the growth of a normal complement of axons to their appropriate target nuclei. One aspect of this question concerns the rather discrete pathways of the central nervous system with bilateral projections—that is, those in which some of the component axons cross the midline axis while others remain on the same side. The optic decussation of mammals provides a good model of such a pathway because the normal complement of crossed and uncrossed axons has been well defined for many mammalian species.

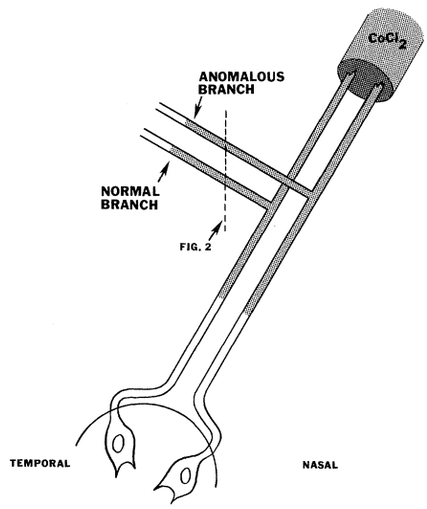


Fig. 1. Schematic diagram showing the site of cobalt injection and both the normal and anomalous uncrossed projections becoming filled, as would be expected if these projections from the retina were formed by branches of crossed axons. The dashed line through the ipsilateral optic tract illustrates the location of the section shown in Fig. 2.

In the normal hooded rat, part of each retina (the peripheral temporal segment) projects to restricted regions of both the left and right subcortical visual centers (*1*). Recent anatomical and electrophysiological studies have shown that a substantial component of these normal bilateral retinal projections consists of ganglion cells whose axons branch, presumably at the optic chiasma, to supply both sides of the brain (*2*). If one eye is removed from the rat at birth, the whole remaining retina now appears to project bilaterally. The result is an expanded distribution of the uncrossed pathway from the remaining eye; instead of the normal restricted distribution in the visual centers the uncrossed pathway occupies almost the total volume of these nuclei, although this projection is not as dense as the remaining crossed projection (*3*). These observations have indicated that there is some modification of the growth of remaining ganglion cell axons at the optic chiasma. The fibers which are normally restricted to the contralateral side have either: (i) been redirected to the ipsilateral side, or (ii) formed atypical uncrossed branches.