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Immunoassay of muscle-specific creatine kinase with a monoclonal antibody and application to myogenesis and muscular dystrophy

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1. A competition e.l.i.s.a. (enzyme-linked immunosorbent assay) is described that enables direct measurement of the muscle-specific polypeptide of chick creatine kinase (M-CK) in extracts of differentiating muscle-cell cultures and in blood plasma samples, even in the presence of embryonic, or brain-type, creatine kinase. The characteristics of the assay can be considerably improved by the use of a monoclonal antibody, CK-ART, instead of rabbit antisera, and we offer an explanation for this in terms of heterogeneity of antibody affinities in polyclonal antisera. In addition to native enzyme, the assay will measure creatine kinase unfolded and inactivated by 8 M-urea treatment. 2. During chick muscle differentiation *in vitro*, M-CK increased from 7.5% of the total creatine kinase at 24 h to 76.0% at 143 h, in good agreement with isoenzyme separation data. As a percentage of the total cell protein, M-CK increased by 156–340-fold over the same period and constituted 0.38–0.56% of the total protein in late cultures. 3. E.l.i.s.a. measurements on 17–20-day embryonic thigh-muscle extracts, which contain almost exclusively M-CK, agree well with enzyme activity and radioimmunoassay. M-CK constituted 0.7–1.6% of the total protein in 17–19-day embryonic thigh muscle. 4. Plasma M-CK concentrations in normal 2–8-week-old chickens were found to be in the range 0.5–0.9 μg/ml. Plasma concentrations of 32–56 μg/ml were found in 8-week-old dystrophic chickens by both e.l.i.s.a. and enzyme-activity measurements. 5. The results suggest that inactive or unfolded forms of M-CK do not normally exist, in any significant amounts, in cell and tissue extracts or in freshly prepared samples of plasma.

Creatine kinase is a dimeric enzyme that exists in two principal isoenzymic forms, the MM-CK dimer in adult skeletal muscle and the BB-CK dimer in other cell types, including brain and early embryonic muscle. The heterodimer, MB-CK, exists in mammalian heart muscle and, transiently, during the differentiation of chick skeletal muscle, when the muscle-specific gene is activated and the synthesis of the muscle-specific polypeptide of creatine kinase (M-CK) begins. M-CK concentrations can be a useful index of muscle differentiation in cell cultures (Morris *et al.*, 1976*a*; Morris, 1978). The concentration of M-CK in blood is widely used in the diagnosis of the X-chromosome-linked muscular dystrophies (Pearce *et al.*, 1964*a*) and in the detection of female carriers (Pearce *et al.*, 1964*b*).

Enzyme-activity measurements, however, suffer from several disadvantages. The presence of embryonic, or brain-form, creatine kinase (B-CK) necessitates preliminary separation of isoenzymes, especially in the early stages of chick myoblast differentiation, when quite large increases in the dominant BB-CK dimer occur at the same time as the appearance of the muscle-specific forms (Morris *et al.*, 1976*a*; Caravatti *et al.*, 1979). The activity of the enzyme can be altered by the presence of ions and other molecules in blood and cell extracts and by the oxidation of essential thiol groups (Watts, 1973; Morin, 1977; Cho & Meltzer, 1979). Other enzymes, such as adenylate kinase, can also interfere in some types of creatine kinase assay (Shainberg *et al.*, 1971).

Immunological assays, using antibodies specific

Abbreviations used: M-CK, muscle-specific subunit of creatine kinase; B-CK, brain-type subunit of creatine kinase; MM-CK, muscle-specific homodimer; BB-CK, brain homodimer; MB-CK, intermediate-form heterodimer; Ig, immunoglobulin; SDS, sodium dodecyl sulphate; e.l.i.s.a., enzyme-linked immunosorbent assay.

for M-CK, overcome these disadvantages and, since the antibodies are usually species-specific, can also be used in the presence of M-CK from other species (e.g. to measure chick creatine kinase in culture medium containing horse or calf serum). Competition e.l.i.s.a. (Voller *et al.*, 1978) has the additional advantages of speed (less than 5h) and the use of stable, non-radioactive reagents.

In the present paper we show that, as a proportion of the total cell protein, M-CK concentrations can increase at least 300-fold during myogenesis *in vitro* and that results with the e.l.i.s.a. agree well with combined enzyme-activity and isoenzyme studies. The characteristics of the e.l.i.s.a. are considerably improved by using the monoclonal antibody CK-ART (Morris & Head, 1982) instead of rabbit antisera, an effect that appears to be due to the presence of low-affinity antibody components in the antiserum. Since the e.l.i.s.a. is shown to measure inactive and unfolded forms of M-CK as well as native enzyme, the results suggest that there are no significant amounts of irreversibly inactivated M-CK in differentiating muscle cells, developing muscle tissue or circulating blood, since good agreement is obtained between e.l.i.s.a. values and enzyme-activity values for M-CK in all cases.

Experimental

Materials

Microtitre plates for e.l.i.s.a. were obtained from Dynatech, Billingshurst, West Sussex, U.K. (96-well M25 or M129B). Peroxidase-conjugated rabbit anti-mouse Ig was obtained from DAKO Antisera (Mercia-Brocades Ltd., West Byfleet, Weybridge, Surrey, U.K.), and peroxidase-conjugated goat anti-rabbit IgG from Miles Laboratories, P.O. Box 37, Stoke Poges, Slough, Berks., U.K. Creatine kinases from rabbit muscle and ox heart were obtained from Sigma, Poole, Dorset, U.K. Rat and mouse thigh-muscle creatine kinases were prepared by the method of Eppenberger *et al.* (1967).

Cell culture

Myoblast cultures were prepared from 12-day embryonic thigh muscle by mechanical dissociation (Tepperman *et al.*, 1975) and grown as previously described (Morris *et al.*, 1976*b*). Creatine kinase activity was determined by a direct fluorimetric method (Morris & Cole, 1972) and total protein by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Antibody production

Chick MM-CK was prepared from 19-day embryonic chick thigh muscle by the method of Eppenberger *et al.* (1967). Antiserum was raised in a male New Zealand White rabbit by an initial multi-site intradermal injection of 1mg in complete Freund's adjuvant and subsequent subcutaneous injections of 1mg in incomplete adjuvant at monthly intervals (Galatowicz & Morris, 1981). Serum from the fourth and fifth bleedings only were used in the present studies. Preparation of the monoclonal antibodies CK-ART and CK-JOE and their purification on creatine kinase–Sepharose 4B affinity columns was as described previously (Morris & Head, 1982). Both belong to the IgG1 subclass, as determined with subclass-specific second antibodies.

The two hybridoma cell lines were obtained by polyethylene glycol fusion of NS1-Ag4/1 myeloma cells with spleen cells from a Balb/c mouse immunized with chick MM-CK. Culture supernatants were screened for anti-(MM-CK) antibody production by e.l.i.s.a., and antibody-producing hybrid cells were cloned twice by limiting dilution (Morris & Head, 1982).

Urea treatment

Creatine kinase was treated with 8.1M-urea by adding 9vol. of 9M-urea and incubating at 37°C for 10min. As the tube was transferred to an ice bath, excess ice-cold e.l.i.s.a. incubation buffer containing 0.5mg of ovalbumin (BDH, Poole, Dorset, U.K.)/ml was added to give a final concentration of 1μg of creatine kinase/ml and less than 0.2M-urea. All subsequent steps were carried out at 4°C. Both enzyme activity and radioimmunoassay data show that 85–90% of the creatine kinase is maintained in an inactive, conformationally altered state by this treatment (Galatowicz & Morris, 1981). We use the term 'unfolded creatine kinase' to describe creatine kinase subjected to this treatment, without implying that the loss of secondary structure is necessarily complete.

Competition e.l.i.s.a.

Microtitre plates were coated with chick MM-CK (0.15ml per well of a 15μg/ml solution in 0.1M-$NaHCO_3$, pH9.6) for 1h at room temperature. The coating solution can be re-used many times and coated plates can be stored for several months at 4°C in sealed bags. The plate was then rinsed twice by flooding the plate with incubation buffer (0.9% NaCl/25mM-sodium phosphate (pH7.2)/0.05% Triton X-100) and flicking away the contents. The plate was then blocked by filling the wells with ovalbumin (10mg/ml) in incubation buffer for 10min and then washed three times with incubation buffer.

While the plate was being coated, antibody and competitor creatine kinase (standards or unknowns) were preincubated together for 1h on a separate 96-well plate in a final volume of 0.15ml of incubation buffer with ovalbumin (0.5mg/ml) and then transferred to the coated and blocked plate for a further 2h incubation.

After being rinsed three times with incubation buffer, the plate was incubated for 1h with peroxidase-conjugated second antibody (1:1000; 0.15ml/well) in incubation buffer with ovalbumin (0.5mg/ml).

After the plate had been rinsed four times with incubation buffer, 0.15ml per well of freshly-prepared substrate [o-phenylenediamine (0.4mg/ml) (Sigma)/0.012% H_2O_2/50mM-Na_2HPO_4/25mM-citric acid] were added for 20–30min before stopping the reaction with 0.05ml of 3M-H_2SO_4.

Each well was diluted with 2ml of water in order to determine the A_{492} spectrophotometrically. The characteristics of the assay were in no way improved by using longer incubation times (up to 24h) for either the coating step or incubations with antibodies. Shorter incubation times have not been studied.

Results

The competition e.l.i.s.a. was carried out in four steps: (1) creatine kinase antigen was allowed to bind to the surface of the plastic microwells; (2) a limiting amount of anti-(creatine kinase) antibody was allowed to bind to this creatine kinase; (3) bound antibody was measured by incubation with peroxidase-conjugated antibody against the appropriate IgG; (4) a colorimetric assay for peroxidase was performed. By carrying out the second step in the presence of competitor creatine kinase, a standard competition curve is obtained from the decrease in bound peroxidase activity.

Fig. 1 illustrates the determination of appropriate antigen and antibody concentrations for the e.l.i.s.a. Coating the plate with 15μg of creatine kinase/ml gave a high maximum level of subsequent antibody binding, so that choice of a limiting antiserum dilution (1:2000–1:3000) still gave a final A_{492} of about 0.5. Coating concentrations as low as 6μg/ml could be used, but concentrations lower than 1.5μg/ml are clearly unsuitable (Fig. 1).

We were unable to detect any enzymic activity in the creatine kinase bound to the plate, even though the assay would have detected 50pg of active enzyme and over 2μg per well were used in the coating step. However, by extracting the wells with either 8M-urea or 1% SDS and then diluting at 37°C, we were able to recover 1.5ng and 1.2ng of active enzyme respectively (results not shown), showing that, although a measurable amount of creatine kinase is bound to the plate, less than 5% of the bound creatine kinase (and perhaps none at all) is enzymically active. Although dilutions of urea-inactivated creatine kinase remain inactive at 4°C (Galatowicz & Morris, 1981), they do slowly renature and partially re-activate at 37°C.

Fig. 2 shows a standard competition curve

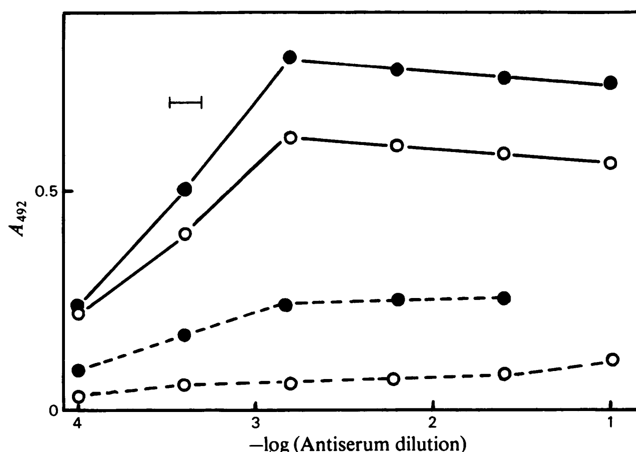

Fig. 1. *Effect of coating creatine kinase concentration and antiserum dilution on anti-(creatine kinase) antibody binding to microtitre plates*

Wells were coated with 15 (●——●), 6 (○——○), 1.5 (●----●) or 0.375 (○----○) μg of chick MM-CK/ml. Serial dilutions (starting at 1:10) of rabbit antiserum were used as the first antibody step in the e.l.i.s.a. as described in the Experimental section. Each point represents a single determination. The horizontal bar (——) indicates the antiserum dilution used in subsequent assays.

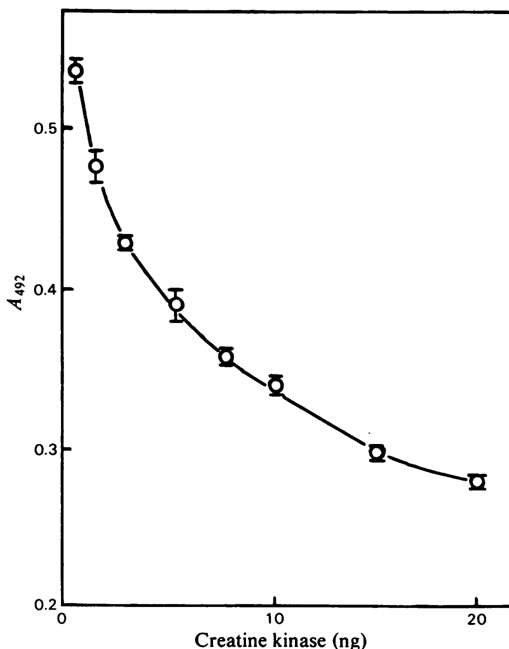

Fig. 2. *Competition e.l.i.s.a.: standard curve*

Bars indicate the S.E.M. for triplicate determinations for each concentration of competitor (chick MM-CK). Rabbit antiserum was used at 1:2000. Full details are given in the Experimental section.

obtained by using rabbit anti-(creatine kinase) serum and purified MM-CK as competitor. The curve characteristically shows a sharp initial fall (down to 45–55% of control), which gives a useful assay range in Fig. 2 of 1–20ng, comparable with the range of a radioimmunoassay with the same antiserum (Galatowicz & Morris, 1981). Increasing the amount of competitor creatine kinase up to 1000ng, however, produced only a further 10% decline in antibody binding. This high 'background' is not due to non-specific IgG binding, since readings of less than 2% of control were obtained when pre-immune serum replaced the antiserum and when creatine kinase was omitted from the coating step (results not shown). It suggests rather that some antibody components of the antiserum with a low affinity for the native creatine kinase competitor are binding to the creatine kinase coat on the e.l.i.s.a. plate.

This interpretation is supported by the marked improvement in the characteristics of the competition curve when rabbit antiserum is replaced by a monoclonal antibody against MM-CK (Fig. 3). The monoclonal antibody used, CK-ART, has the same properties, in all tests performed so far, as those of CK-JOE described previously (Morris & Head, 1982), except that its apparent functional affinity for creatine kinase is 10–20 times higher in both radioimmunoassay and competition e.l.i.s.a. This is illustrated in Fig. 4(*a*), which shows that, at identical

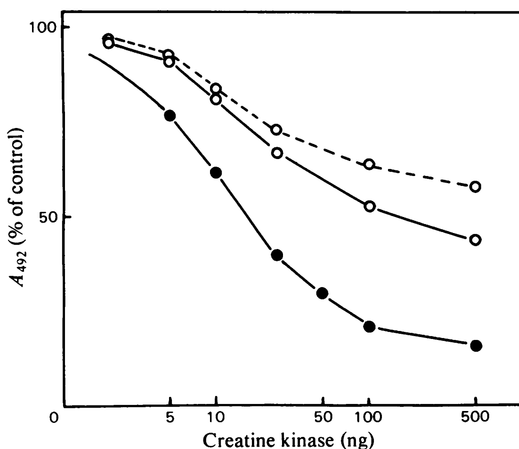

Fig. 3. *Comparison of competition e.l.i.s.a. standard curves using 1:2000 rabbit antiserum* (○- - - -○), *affinity-purified rabbit antibodies from this antiserum* (○——○), *and the monoclonal antibody, CK-ART* (●——●)

Each point represents the mean of duplicate determinations. Antibody concentrations were chosen by a preliminary direct e.l.i.s.a. (Fig. 1) to give approximately the same A_{492}, and results were normalized to 100% in the absence of competitor creatine kinase.

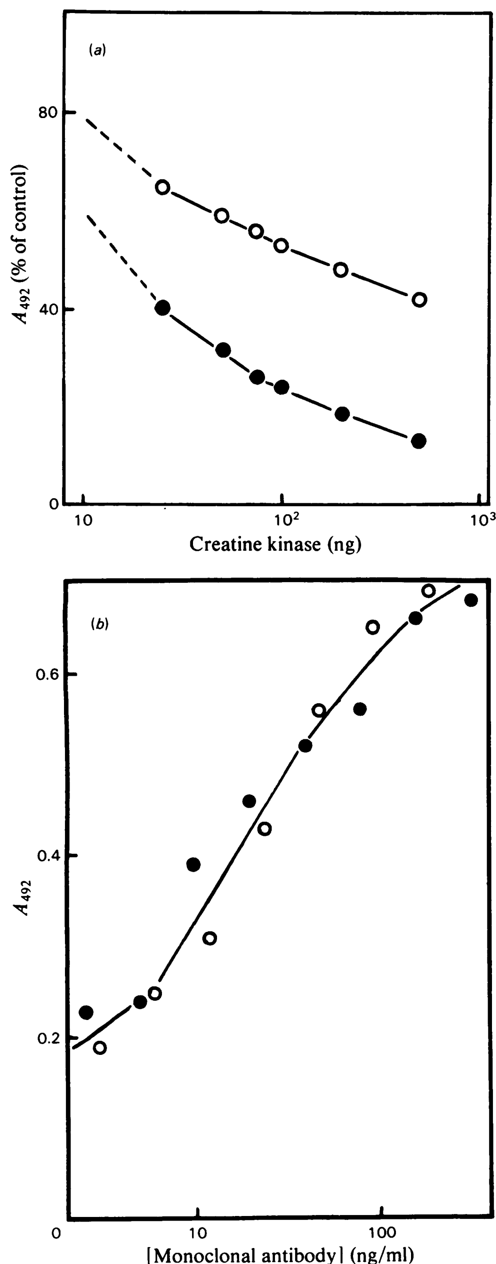

Fig. 4. *Effect of antibody affinity on e.l.i.s.a. with monoclonal antibodies*

(*a*) Comparison of competition e.l.i.s.a. standard curves using CK-ART (●——●) and CK-JOE (○——○). Protein concentrations of monoclonal antibodies (affinity-purified on creatine kinase–Sepharose 4B columns) were determined by the method of Lowry *et al.* (1951) and the antibodies were used at 100ng/ml. Each point represents the mean of duplicate values. (*b*) Comparison of the direct binding of CK-ART (●) and CK-JOE (○) to the creatine kinase-coated e.l.i.s.a. microtitre plate. Each point represents a single determination.

monoclonal-antibody-concentration, 15–20 times more competitor creatine kinase is required with CK-JOE to produce the same competition as in the assay with CK-ART. In contrast, there is very little difference between the binding of the two monoclonal antibodies to the creatine kinase-coated plate in the absence of competitor (Fig. 4*b*). The latter experiment was repeated twice and the difference between the two curves was never more than 2-fold, confirming the commonly held view that solid-phase antibody binding is relatively independent of affinity (Hudson & Hay, 1980). It is clear from these results that the presence in anti-(creatine kinase) serum of antibody components with affinities similar to that of CK-JOE could produce the kind of competition curve seen in Figs. 2 and 3.

Two further observations are also consistent with this interpretation. First of all, use of affinity-purified anti-(creatine kinase) Ig from the same antiserum produces only a slight improvement in the competition curve (Fig. 3). Secondly, e.l.i.s.a. plates coated with various mammalian creatine kinases [rabbit and ox (Fig. 5); rat and mouse (not shown)] do bind Ig from the antiserum but do not bind CK-ART or CK-JOE. In affinity-dependent *competition* assays (e.g. radioimmunoassay) mammalian creatine kinases do not compete at all with chick creatine kinase for either antisera or monoclonal antibodies (results not shown). Together these observations are consistent with the presence in the antiserum of low-affinity antibodies against creatine kinase, some of which cross-react with mammalian creatine kinases. A further disadvantage of antisera as opposed to monoclonal antibodies is that we have observed considerable variation in the sensitivity of the e.l.i.s.a. at low competitor creatine kinase concentration (cf. Figs. 2 and 3). We cannot fully explain this, but a change in the shape of the competition curve towards that of Fig. 2 can be obtained by diluting the antisera. The curve still tends towards a plateau at 50–60% competition (results not shown).

Fig. 6 shows that creatine kinase unfolded by treatment with 8 M-urea is just as effective a competitor in the e.l.i.s.a. as native creatine kinase. Under the same conditions of urea treatment, 85–90% of the enzymic activity and reactivity in a radioimmunoassay (with rabbit antiserum) were destroyed (Galatowicz & Morris, 1981).

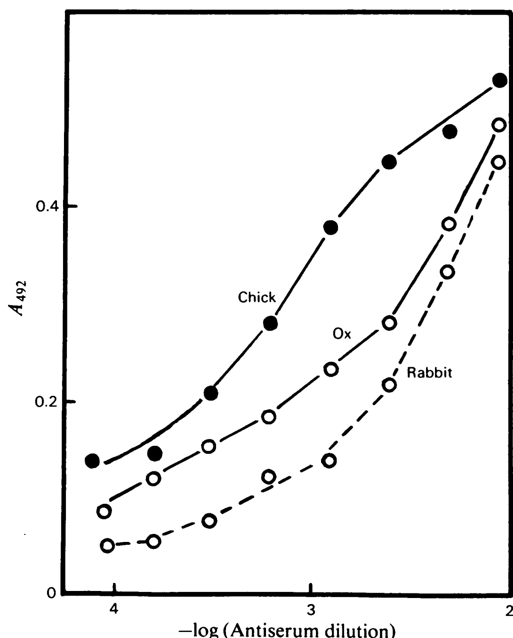

Fig. 5. *Binding of antibodies from rabbit antiserum to microtitre plates coated with MM-CKs from different species*

Wells were coated with chick (●——●), ox (○——○) or rabbit (○– – – –○) MM-CK species, each at 15 μg/ml and then subjected to direct e.l.i.s.a. as described in Fig. 1, serial dilutions of rabbit antiserum being used. Each point represents a single determination.

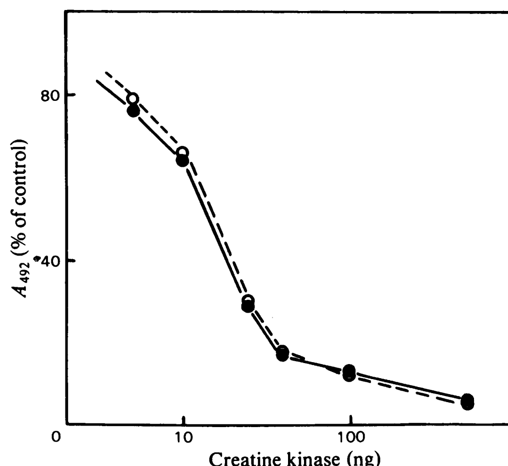

Fig. 6. *Effect of urea treatment on the ability of MM-CK to act as competitor in the e.l.i.s.a.*

Urea-treated creatine kinase was prepared as described in the Experimental section. To prevent renaturation of urea-unfolded creatine kinase, both control (●——●) and urea-treated (○– – – –○) MM-CK were maintained at 4°C in a cold-room during all e.l.i.s.a. steps in which they were present as competitor. Other steps were carried out at room temperature as usual. There was no apparent effect of the lower temperature on the control e.l.i.s.a. Each point represents the mean of triplicate determinations. CK-ART was used in this experiment as a hybridoma culture supernatant at 1:500 dilution. Similar results were obtained when 1:2000 rabbit antiserum was used (results not shown).

Table 1. *Determination of M-CK concentration in extracts of chick embryonic thigh muscle by enzyme activity, e.l.i.s.a. and radioimmunoassay (RIA)*

The higher creatine kinase concentrations in Expts. 2 and 4 simply reflect higher extraction efficiencies (no attempt was made to achieve complete extraction). Each value is the mean of three or four determinations on the same extract. Extracts were prepared by homogenization in 0.1% Triton X-100/5 mM-2-mercaptoethanol/0.9% NaCl/25 mM-sodium phosphate, pH 7.2 (10 ml/four thighs for 17- and 18-day embryo; 10 ml/two thighs for 19- and 20-day embryo) followed by centrifugation at 15000 *g* for 15 min to remove insoluble material.

		Creatine kinase concentration (μg/ml) by:					
Expt. no.	Age of embryo (days)	Enzyme activity (*a*)	E.l.i.s.a. (CK-ART) (*b*)	E.l.i.s.a. (antiserum) (*c*)	RIA (CK-ART) (*d*)	RIA (antiserum) (*e*)	$\frac{(b)}{(a)} \times 100$
1	17	135	190	—	—	—	141
	19	170	250	—	—	—	147
2	18	354	300	—	—	—	85
	20	574	400	—	—	—	70
3	18	190	144	143	139	135	76
	19	173	136	133	124	131	79
4	18	445	510	440	400	300	107
	20	430	550	290	570	480	128
							Mean: 103 ± 11

Embryonic chick thigh muscle between 17 and 20 days of incubation *in ovo* has 95–99% of its creatine kinase as the M-form (Stewart *et al.*, 1981). In muscle tissue extracts, the e.l.i.s.a. should therefore give the same results as enzyme assay of total creatine kinase content. The results in Table 1 confirm this. In four experiments with eight different extracts, the average e.l.i.s.a. value for M-CK as a percentage of the enzyme activity value for total creatine kinase was 103 ± 11 (S.E.M., eight observations). Table 1 also shows that similar results were obtained by radioimmunoassay and when CK-ART monoclonal antibody was replaced by rabbit antiserum.

Fig. 7 shows a comparison of the time course of accumulation in differentiating myoblast cultures of M-CK (by e.l.i.s.a.) and total creatine kinase (by enzyme activity), both expressed in absolute terms by reference to a purified MM-CK standard [purified MM- and BB-CK species may differ slightly in activity (Eppenberger *et al.*, 1967), but no attempt has been made to correct for this]. The increase in M-CK between 24 and 143 h is from 11 ng to 3750 ng per mg of total protein (340-fold), or from 2 ng to 7890 ng per culture dish, or from 7.5 to 76.0% of the total creatine kinase (MM, MB, BB) in the cultures. In a second similar experiment, M-CK increased 156-fold from 36 ng/mg to 5600 ng/mg of protein between 24 and 115 h, but attained the same final proportion of 76% M-CK. Immunofluorescence microscopy, however, reveals that most, if not all, of the M-CK in 24 h cultures is present in the very small number of myotubes which survive the myoblast preparation procedure and attach to the culture dish (G. E. Morris, unpublished work; cf. Dawkins & Lamont, 1971; Turner *et al.*, 1976*a*). The increases in M-CK observed in bulk cultures, whether 156-fold or 340-fold, are therefore considerable underestimates of the change in expression of the M-CK gene as M-CK protein during myoblast differentiation. The increase in B-CK between 24 and 42 h, which is evident in Fig. 7, was found previously by isoenzyme separation methods (Morris *et al.*, 1976*a*; Caravatti *et al.*, 1979). The difference between the two creatine kinase accumulation curves in Fig. 7 should reflect the relative amounts of M-CK and B-CK at different stages of differentiation. This is confirmed by the results in Table 2, which show that the percentage of M-CK from Fig. 7 agrees well with our own previously published isoenzyme separation data.

In order to compare these M-CK concentrations in 5–6-day cultures from 12-day embryonic muscle with M-CK concentrations in muscle *in vivo*, we measured creatine kinase in homogenates of 17-day and 19-day chick-embryo thigh muscles. The concentration *in vivo* of 0.7–1.6% of total protein (Table 3) is rather higher than in the cultures (0.38–0.56%).

Although these results establish the credentials of the e.l.i.s.a. for cell and tissue extracts it is necessary to establish its applicability to creatine kinase concentrations in blood samples, which contain a quite different set of proteins, including chick Ig. The results in Table 4 confirm the large increase in plasma creatine kinase concentrations in dystrophic chickens between 2 and 8 weeks post-hatching (Barnard & Barnard, 1979), which does not occur in birds of the control strain. Once again there is good agreement between e.l.i.s.a. and enzyme-activity data, since plasma creatine kinase in dystrophic chickens is almost entirely M-form (Stewart *et al.*, 1981). Preliminary experiments with stored or frozen

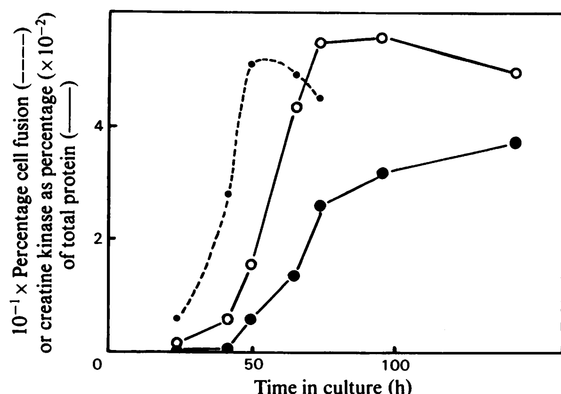

Fig. 7. *Accumulation of total creatine kinase* (○——○) *and muscle-specific M-CK* (●——●) *during differentiation of muscle cells in culture*

Culture plates (in triplicate) were taken at different culture times, rinsed in ice-cold saline (0.9% NaCl/25 mM-sodium phosphate, pH 7.2) and stored at −20°C. Cultures were later thawed, harvested in ice-cold 0.9% NaCl/25 mM-sodium phosphate (pH 7.2)/0.5% Triton X-100, and centrifuged at 2000 *g* for 10 min. Supernatants were diluted either in TM buffer (Morris & Cole, 1972) with 0.5 mg of bovine serum albumin/ml for enzyme assay or in e.l.i.s.a. incubation buffer with 0.5 mg of ovalbumin/ml. Total protein (Lowry *et al.*, 1951) was determined on samples of cell extracts (taken before centrifugation) with 1% SDS present to prevent Triton precipitation. Results are expressed as percentages of total protein. Percentage cell fusion (●----●) in the cultures was determined as described previously (Morris & Cole, 1972).

Table 2. *Determination of M-CK as a percentage of total creatine kinase in myogenic cell cultures by e.l.i.s.a. and by isoenzyme separation*

The e.l.i.s.a. data are obtained from Fig. 7, and isoenzyme-separation data from the following sources: [1]Morris & Cole (1979); [2]Morris *et al.* (1976*c*); [3]Morris *et al.* (1976*a*).

Time in culture (h)	M-CK calculated by e.l.i.s.a. as a percentage of total creatine kinase calculated by enzyme activity	Percentage of M-CK calculated by isoenzyme separation[1]
24	7.5	—
42	10.0	14[2], 13[3]
50	36.5	—
66	31.5	—
74	47.8	46[3]
96	60.0	61[1], 68[2], 54[3]
143	75.6	—

plasma suggest that, under some circumstances, creatine kinase in plasma can lose enzyme activity without loss of immunological activity in the e.l.i.s.a., which may therefore be especially useful for assaying samples that have not been collected or stored under optimal conditions.

Table 3. *Determination of creatine kinase concentration in 17-day and 19-day embryonic thigh muscle*

Four separate homogenates were prepared in 10 ml of 0.1% Triton X-100/5 mM-2-mercaptoethanol/0.9% NaCl/25 mM-sodium phosphate, pH 7.2, two each from 17-day embryos (four thighs/10 ml) and 19-day embryos (two thighs/10 ml). Creatine kinase enzyme assays (Morris & Cole, 1972) were performed in duplicate on 1:5000 or 1:10000 dilutions of the homogenates in TM buffer [100 mM-Tris/acetate (pH 6.8)/25 mM-magnesium acetate] plus 1 mg of bovine serum albumin/ml. Portions (5 μl) of homogenate were assayed in duplicate for total protein (Lowry *et al.*, 1951). Differences between duplicates were less than 3% for both assays.

Age of embryo (days)	Creatine kinase concentration in homogenate (μg/ml)	Total protein concentration in homogenate (μg/ml)	Creatine kinase as a percentage of total protein
17	74	11400	0.65
	76.5	10500	0.73
19	185	15000	1.23
	235	15000	1.57

Table 4. *Determination of plasma creatine kinase concentrations in normal and dystrophic chickens by competition e.l.i.s.a. and by enzyme assay*

Each value is the mean of four determinations on a fresh plasma sample from a single bird. Chickens were the Imperial College London strains of normal (line 412) and dystrophic (line 413) birds, derived from the University of California lines (Barnard & Barnard, 1979). The two values for each measurement represent two different birds.

Age (weeks)	Condition	[Plasma creatine kinase] (μg/ml) E.l.i.s.a.	Enzyme assay
2	Normal	0.90, 0.52	1.08, 0.78
	Dystrophic	0.96, 0.98	1.20, 1.28
8	Normal	0.62, 0.52	0.88, 0.95
	Dystrophic	56.0, 31.5	50.5, 32.3

Discussion

One purpose of the present paper is to show that M-CK concentrations in muscle and plasma can be determined in a direct assay without the necessity of separating isoenzymes or even of preserving the active or native conformation of the enzyme. Few previous measurements of creatine kinase in myoblast cultures have used immunological assays, though a micro-complement-fixation assay has been performed (Turner *et al.*, 1976*b*) and antibodies have been used to separate isoenzymes before enzymic assay (Perriard *et al.*, 1978). We have

ourselves described a suitable radioimmunoassay using rabbit antisera (Galatowicz & Morris, 1981), though this, like most such assays of globular proteins (Crumpton, 1974), measures antigen in its native conformation only. The e.l.i.s.a. measures most, if not all, enzymically inactive forms of M-CK, so there is no evidence from the present studies for the presence of such inactive forms in muscle cells, embryonic muscle tissue or circulating blood (Tables 1, 2 and 4). Since all comparisons were made against our purified chick MM-CK standard, we can only say strictly that the proportion of M-CK that is active in intact muscle is the same as in our purified preparation. However, it seems rather unlikely that a constant proportion of the total M-CK would be fortuitously inactivated in the course of preparation of both the samples and the two separate MM-CK standards used in the course of this work. Two forms of M-CK have been described in both chick and rat, differing in isoelectric point, synthesized in approximately equal amounts and possibly the products of different genes (Rosenberg *et al.*, 1981), but both forms appear to be enzymically, as well as immunologically, active.

The increase in M-CK of over 300-fold during myogenesis *in vitro* (Fig. 7) is a useful indicator of the quality of bulk cultures of myoblasts as a biochemical 'model' for myogenesis, though it must be a considerable underestimate of the true extent of the differentiation process because of the presence, in 24h cultures, of a few myotubes with very high M-CK concentrations. There is no reason to suppose that there is any M-CK in dividing myoblast precursor cells (Turner *et al.*, 1976*a*).

Perhaps a better indication of the extent of differentiation in culture is the final concentration of M-CK of 0.4–0.6% of the total protein, a value which agrees well with [^{3}H]leucine-incorporation studies of newly-synthesized M-CK as a proportion of the total newly-synthesized protein in myotube cultures (Morris & Cole, 1977). The value also compares reasonably favourably with the 0.7–1.6% creatine kinase content of 17–19-day embryonic muscle (Table 3). Concentrations *in vitro* and *in vivo* of other muscle-specific proteins, such as the Ca^{2+}-transporting ATPase (Martonosi *et al.*, 1979), are also comparable during this period, though further increases do occur *in vivo* at later stages. Of course, the composition of the other muscle proteins *in vivo* may be too different from that in cultures for the comparison to be strictly valid.

We have shown that the e.l.i.s.a. with the monoclonal antibody CK-ART is capable of measuring M-CK in plasma from normal and dystrophic chickens, with no evidence of interference by other plasma proteins (Table 4). With a suitable monoclonal antibody against human M-CK, the assay could clearly be applied to diagnosis of Duchenne muscular dystrophy and to detection of female carriers of the X-chromosome-linked gene (Pearce *et al.*, 1964*a*,*b*). Loss of enzyme activity by thiol oxidation or other mechanisms is known to be a problem in such diagnostic tests on human serum (Cho & Meltzer, 1979; Morin, 1977). Variation between individual carriers and the high scatter of creatine kinase values about the mean for the normal population makes it impossible to detect all carriers with confidence using present methods. An e.l.i.s.a. might possibly improve matters, depending on the extent to which variations are due to differential enzyme inactivation between serum samples, rather than real differences in rates of creatine kinase release from muscle.

The ability of the competition e.l.i.s.a. to measure unfolded, as well as native, M-CK (Fig. 6) is due both to the solid-phase e.l.i.s.a. itself (since even rabbit antisera measure unfolded creatine kinase in this assay) and to the monoclonal antibody (since CK-ART measures unfolded creatine kinase even in the radioimmunoassay) (G. E. Morris & L. P. Head, unpublished work). The epitope on M-CK recognized by CK-ART thus appears either to require no secondary structure, or to have an unusually highly ordered conformation which is not altered by 8M-urea or re-forms very readily after dilution of the urea, even at 4°C.

A possible explanation for the ability of rabbit antisera to measure unfolded creatine kinase in the e.l.i.s.a., but not in the radioimmunoassay, may lie in the fact that antisera are usually heterogeneous mixtures of antibodies with different affinities and specificities, so that the e.l.i.s.a. could, in theory, involve different component antibodies from radioimmunoassay. It has long been recognized that solid-phase antibody-binding assays like direct e.l.i.s.a. do not show the strong affinity-dependence characteristic of radioimmunoassays in solution (Hudson & Hay, 1980), though there clearly must be some minimum affinity for antibody binding to occur (Butler *et al.*, 1978). The availability of two monoclonal antibodies (CK-ART and CK-JOE) differing only in affinity for M-CK has enabled us to illustrate this lack of dependence on affinity in a simple way (Fig. 4*b*), without the interpretative difficulties that are inevitable when one uses heterogeneous polyclonal antisera. The *competition* e.l.i.s.a. is affinity-dependent (Fig. 4*a*).

The failure of larger amounts of creatine kinase to achieve complete competition in the e.l.i.s.a. (Figs. 2 and 3) is only the first indication that the antiserum contains antibody components of lower affinity. The second is the binding of antiserum Ig to plates coated with mammalian creatine kinases, which do not cross-react in radioimmunoassays (Fig. 5). A further indication comes from the effects of urea-unfolding. Creatine kinase bound to an e.l.i.s.a. plate

is enzymically inactive, probably as a result of losing its native conformation. This explanation is supported by the observation that the e.l.i.s.a. plates can be coated with urea-unfolded creatine kinase with little effect on the ability of the assay to measure either native or unfolded enzyme (G. E. Morris & L. P. Head, unpublished work). Alternative possibilities are that binding prevents substrate access to the catalytic site or that multi-site binding to the plate prevents the enzyme from undergoing its essential conformational changes on binding its substrates (Watts, 1973); but, if the creatine kinase were bound in its native form, it would be difficult to explain why an antiserum that is specific for native conformation in a radioimmunoassay should show no such specificity in the e.l.i.s.a. (Fig. 6). A simpler explanation is possible if we suppose that the creatine kinase bound to the plate is not in its native form, so that different antibody components could be involved in the two assays. Thus, because of the Law of Mass Action, a high-affinity component specific for *native* creatine kinase could monopolize the radioimmunoassay (Butler, 1980), whereas other components of lower affinity, but which bind both native and unfolded creatine kinase equally, could participate in the e.l.i.s.a. Further monoclonal-antibody studies should provide evidence for or against this line of argument. Monoclonal antibodies specific for native creatine kinase in a radioimmunoassay should now be sought so that their behaviour in e.l.i.s.a. assays can be studied directly. Whatever the explanation, the results clearly show that antisera may display different specificities in different types of assay, whereas monoclonal antibodies are, predictably, more consistent in this respect.

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