of arginine in the hydrolysate, which corresponds to 1 μmole of pepsin[11]. Thus DDE-inhibited pepsin contains one inhibitor residue per protein molecule.

It is obvious that the manner of DDE action on pepsin strongly resembles that of diazoacetyl-DL-norleucine methyl ester which also combines with enzyme in a 1:1 ratio[2]. We found that DDE did not interact with hog pepsin previously inhibited with diazoacetyl-DL-norvaline methyl ester — a close homologue of diazoacetyl-DL-norleucine methyl ester.

DDE can also be used for inhibition or chemical modification of other enzymes. Thus treatment of calf rennin (EC 3.4.4.3) with DDE under the conditions described in this paper for the inhibition of pepsin leads to complete loss of its rennin activity.

Institute for Chemistry of Natural Compounds,
Academy of Sciences of the U.S.S.R.,
Moscow (U.S.S.R.)

V. M. STEPANOV
L. S. LOBAREVA
N. I. MAL'TSEV

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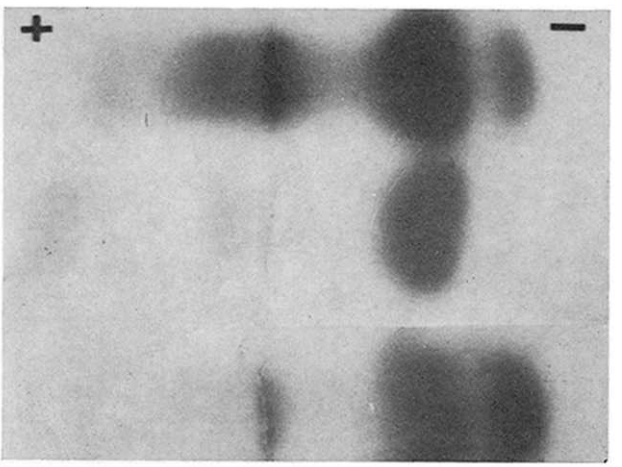
Heterogeneity of pig heart creatine kinase

In addition to the well-established pattern of isoenzymes for soluble creatine kinases (ATP:creatine phosphotransferase, EC 2.7.3.2), recent data support the existence of further heterogeneity at the subcellular level. JACOBS, HELDT AND KLINGENBERG[1] produced the first evidence for the existence of creatine kinase in highly purified mitochondria from skeletal muscle, as well as from heart and brain of the rat. They demonstrated electrophoretically that the enzyme of mitochondrial origin differs from the well known isoenzymes of the soluble fraction. Using guinea-pig brain, SWANSON[2] found differences in kinetic behaviour, both in rates and in pH optima, between crude extracts of the soluble enzyme and that found associated with a mitochondrial suspension. However, he was unable to solubilize this latter enzyme. Further, OTTAWAY[3] has produced evidence for a creatine kinase associated with the myofibrillar fraction of ox heart muscle.

These results prompt us to report the first purification and crystallization, from

TABLE I

DIFFERENTIAL EXTRACTION OF SOLUBLE AND PARTICULATE FRACTION ENZYMES

Expt. No.	*Extract*	*Total units*	*Electrophoretic pattern*†	*Creatine phosphokinase activity (units/ml)*††	
				+	−
1	Whole cell extract*	61 800		336	230
2a	Sucrose extract**	37 400		208	170
2b	Phosphate extract of residue from 2a***	32 166		179	51

* Minced pig heart extracted with 0.1 M phosphate buffer (pH 7.2) containing 10^{-3} M EDTA and 10^{-5} M dithiothreitol.

** Mince homogenized 30 sec with Waring blendor in 2 vol. of cold 0.3 M sucrose.

*** Residue from Expt. 2a re-extracted with 0.1 M phosphate buffer (pH 7.2) containing 10^{-3} M EDTA and 10^{-5} M dithiothreitol.

† 1.0% agar in 0.1 M Tris buffer (pH 9.0) containing 0.1% bovine serum albumin and 10^{-4} M diothiothreitol. Electrophoresis conducted at 10 mA, 300 V for 90 min. For enzyme stain, see ref. 5.

†† Activity determined titrimetrically by the method of MAHOWALD, NOLTMAN AND KUBY[6]. Units expressed as μmoles H^+ produced per min. +, with dithiothreitol; −, without dithiothreitol.

pig heart, of a creatine kinase from a particulate but non-mitochondrial fraction of the cell, as well as purification to homogeneity of the principal cytoplasmic enzyme.

The particulate enzyme is of special interest as it represents the first reported case of creatine kinase with a sedimentation coefficient as high as 11.7—a value consistent with a molecular weight far in excess of the value of 81 000 reported for other creatine kinases. The purified particulate enzyme also shows an absolute requirement for a thiol protective agent whereas the soluble form of the heart creatine kinase shows no such requirement.

The initial resolution was achieved by differential extraction as outlined in Table I, which also shows the agar gel electrophoretic patterns of the various extracts[4].

Sucrose homogenates yield little over half the total activity extracted into phosphate buffer. Re-extraction of the residue with phosphate released the remaining activity, which was shown to be associated with a new electrophoretic species migrating further towards the cathode. This activity was markedly dependent on dithiothreitol. In fact, difficulty was encountered in detecting the enzyme on the gel if dithiothreitol and bovine serum albumin were omitted, even though the soluble enzyme stained readily.

TABLE II

PURIFICATION OF SOLUBLE FRACTION CREATINE KINASE FROM PIG HEART

Step	*Fraction*	$\frac{A_{280\ m\mu}}{A_{260\ m\mu}}$	*Specific activity*†	*Yield (%)*	*Total protein (mg)*
1	Initial sucrose extract*	0.50	5.5	100	19 250
2	50–65% ethanol**	1.19	16.3	65	4 250
3	DEAE-cellulose***	1.89	144.0	71	526

* 850 g mince was homogenized with 2 vol. of 0.3 M sucrose. During the extraction, the pH of the supernatant was adjusted to 7.0 with 1 M NaOH.

** The 1600 × *g* supernatant was made 0.1 M in magnesium acetate and 10^{-4} M in dithiothreitol; temp., −15°. Precipitate was redissolved in 0.01 M *N*-ethylmorpholine (pH 8.3) containing 10^{-3} M EDTA and 10^{-5} M dithiothreitol.

*** DEAE-cellulose, type 40, Brown Co.; column dimensions, 5 cm × 45 cm; the enzyme was eluted with a linear gradient (0–0.1 M NaCl in 1 l). The enzyme eluted at 1.2 $m\Omega^{-1}$ and fractions with an $A_{280\ m\mu}/A_{260\ m\mu}$ ratio of not less than 1.87 were combined.

† Specific activity is expressed as μmoles H^+ produced per min per mg protein at pH 8.8, 30°.

The subsequent purification steps are summarized in Tables II and III.

The specific activity of the soluble enzyme remains unchanged on further chromatography on CM-cellulose (pH 7.0) and is constant throughout the peak fractions. The particulate enzyme crystallized readily at low ionic strengths ($I = 0.01$) as large, extremely thin and flexible square plates. By contrast, the cytoplasmic enzyme was soluble under the same conditions of protein concentration and ionic strength. The large drop in specific activity of the crystallized particulate fraction enzyme appears to be related to the level of salt used to redissolve the crystals. The enzyme is reversibly inactivated by NaCl, a 92% inhibition occurring at a concentration of 0.5 M NaCl.

Both enzymes are homogeneous on agar gel and cellulose acetate electrophoresis (pH 8.6 and 9.0). When examined in the ultracentrifuge, the particulate and soluble fraction enzymes sediment as single symmetrical peaks, but with markedly different sedimentation coefficients (s_{20}=11.73 and 5.10, respectively). In 4 M guanidinium chloride, the s_{20} values fell to 1.35 and 1.10. At this concentration, the number of titratable thiol groups was maximal. Preliminary studies with 5,5'-dithiobis-(2-nitrobenzoic acid) reveal significant differences in both the total number of thiol groups and the number which titrate in the absence of guanidinium chloride.

That the particulate fraction enzyme studied, is not associated with mitochondria was revealed by electrophoretic analysis of subcellular fractions. These were obtained by isopycnic centrifugation for 120 min at 500 000 × *g* through a gradient of 0.4 to 2.0 M sucrose. The enzyme was located at the base of the gradient, and likewise has been shown to be absent from highly purified mitochondria.

The particulate creatine kinase we have described from pig heart appears to resemble, in solubility and cellular distribution, the enzyme described by OTTAWAY[3]. However, the pig heart enzyme has a much higher specific activity and unlike the ox enzyme has been obtained in an electrophoretically pure state. On the basis of electrophoretic evidence, the particulate creatine phosphokinase from pig and ox differ markedly in charge. It is also worthy of note that in the absence of the thiol protective

TABLE III

PURIFICATION OF PARTICULATE FRACTION ENZYME FROM PIG HEART

Step	*Fraction*	$\frac{A_{280\ m\mu}}{A_{260\ m\mu}}$	*Specific activity*	*Yield (%)*	*Total protein (mg)*
1	Phosphate extract*	0.78	4.3	100	11 450
2	25–35% $(NH_4)_2SO_4$ cut**	0.85	8.7	38	4 360
3	DEAE-cellulose***	1.47	165	30	228
4	Crystallization†	1.75	139	27	200

* Residue from Step 1 in Table II was re-extracted with an equal volume of 0.1 M phosphate buffer (pH 7.2) containing 10^{-3} M EDTA and 10^{-5} M dithiothreitol.

** The 25–35% $(NH_4)_2SO_4$ cut contains 38–40% of the total activity, with the particulate enzyme as the major component. The contaminating soluble fraction enzyme is removed in the 55–75% cut.

*** DEAE-cellulose, type 40, Brown Co.; column dimensions, 5 cm × 45 cm. The enzyme was first desalted with Sephadex G-25 equilibrated with 0.01 M *N*-ethylmorpholine (pH 8.3) containing 10^{-3} M EDTA and 10^{-5} M dithiothreitol. Enzyme elutes at 3 mΩ^{-1} in a linear gradient (0–0.2 M NaCl in 2 l).

† Combined enzyme fractions are concentrated to 7 mg/ml protein by evaporation from dialysis tubing, crystallization occurring on dialysis against 0.01 M *N*-ethylmorpholine buffer (pH 7.2) containing 10^{-3} M EDTA and 10^{-5} M dithiothreitol.

agent, the particulate enzyme from pig was not detected on zymograms which showed full development of the enzyme activity for both mitochondrial and soluble fractions.

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Department of Biochemistry,
University of Queensland,
St. Lucia, Queensland, 4067
(Australia)

AILA I. KETO
MARY D. DOHERTY

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