was optimum at pH 4.5 with globin as substrate. Glycyltyrosine amide, hippurylphenylalanine, benzoyloxycarbonylglutamylphenylalanine and to a smaller extent leucine amide were readily hydrolysed by the proteinase, but hippurylarginine and benzoylarginine amide were not. The partially purified protease was very active in releasing NADH-cytochrome *b₅* oxidoreductase from microsomes.

The oxidoreductase released from microsomes was isolated from the high-speed supernatant after digestion by a procedure involving ultrafiltration through a Diaflo XM 50 membrane, chromatography on Sephadex G-100 and isoelectric fractionation in the pH ranges 3–10 or 5–8. The final preparation was enriched 140-fold in oxidoreductase activity over the starting microsomes. The preparation behaved as a single molecular species on polyacrylamide gel and had an isoelectric point of pH 6.5. The enzyme transfers electrons from NADH to cytochrome *c* only in the presence of cytochrome *b₅*.

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Distribution of Arylsulphatase C in Subcellular Membranes of Rat Liver

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Earlier studies from these laboratories have established that arylsulphatase C, one of the arylsulphatase enzymes (arylsulphate sulphohydrolases, EC 3.1.6.1) found in mammalian tissues, is localized in the endoplasmic reticulum in the rat liver cell (Dodgson, Spencer & Thomas, 1954; Dodgson, Rose & Spencer, 1957). Further, the enzyme was found to be firmly bound to the insoluble membrane component of this fraction, and it has been suggested that it might be useful as a microsomal marker enzyme (Milsom, Rose & Dodgson, 1968). However, in routine cell-fractionation studies the nuclear, mitochondrial and lysosomal fraction were always found to possess some arylsulphatase C activity, and it was decided to determine whether this was due solely to contamination with microsomal material.

Nuclei could be readily freed from the enzyme by sedimentation through 2.2M-sucrose by the method of Widnell & Tata (1964). Purification of the mitochondrial and lysosomal fractions by washing five

times with 0.25M-sucrose or by density-gradient centrifugation removed up to 90% of their original activity. That which remained in washed mitochondria was mainly concentrated in the outer-membrane component obtained by the 'shrink' method (Sottocasa, Kuylenskierna, Ernster & Bergstrand, 1967), although the specific activity was only 20% of that of microsomal membranes. The activity of the inner-membrane component was too low to measure accurately.

Lysosomal-membrane material that has been repeatedly washed in iso-osmotic sodium chloride still retained some activity. However, the enzyme was not found in association with tritosomes obtained from rats receiving Triton WR-1339 (Wattiaux, Wibo & Baudhuin, 1963), although the activity of the remainder of the subcellular components was normal.

In all these experiments the distribution of glucose 6-phosphatase closely paralleled that of arylsulphatase C. It is concluded from these studies that both enzymes are most probably localized only in the endoplasmic reticulum of the rat liver cell and that the presence of activity in other membranes represents contamination with this fraction. A more precise determination of the intracellular localization of arylsulphatase C will not be possible until a specific histochemical method for its detection has been developed.

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Further Evidence for the Role of the Essential Thiol Groups in Adenosine Triphosphate-Creatine Phosphotransferase from a Comparison of the Human and Rabbit Enzymes

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Human muscle creatine kinase has two reactive thiol groups and a total of 7–8 thiol groups/molecule just like the rabbit enzyme. It also has two sub-

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units, with closely similar amino acid compositions and peptide 'maps'. The reactive thiol groups have a similar reaction rate with iodoacetamide that is characteristically independent of pH. However, although the effect of single substrates is slight, combinations of substrates alter the inhibition reaction in quite a different way. The most striking difference is with creatine plus Mg-ADP. This combination, shown by O'Sullivan, Diefenbach & Cohn (1966) to give almost complete protection to the rabbit enzyme against inhibition by iodoacetamide, strongly enhances the rate of inhibition of human enzyme. Varying the pH of the reaction shows that the reactive thiol groups have become freely ionizing with a pK near 8.6. Concentrations of this substrate combination that less than half-saturate the enzyme have a complex effect in which, as compared with the control, the reactive thiol groups appear first activated and then protected as the reaction proceeds. Inhibition rarely exceeds 60% under the conditions used, whereas with the rabbit enzyme 90% inhibition is achieved. An equilibrium mixture of substrates shows the same phenomenon, but the active combination is probably creatine plus Mg-ATP.

These results seem to be compatible only with creatine plus Mg-ADP enhancing the activity of the thiol in the subunit to which they bind but causing protection of the reactive thiol group in the subunit without substrate. Some form of co-operatively induced conformational change between the two subunits is indicated, and if it were stabilized by the alkylation of the more reactive thiol group then this would explain why the reaction does not go to completion. The reaction goes much nearer to completion in the presence of high concentrations of creatine plus Mg-ADP, which would simultaneously activate the thiol groups on both subunits.

Unlike the rabbit enzyme no protective effects could be detected that might be interpreted as an interaction of the reactive thiol group with creatine in an intermediate complex of the enzyme reaction. Hence the mechanism of action suggested by Watts & Rabin (1962) is not supported and a more conformational role for the thiol group is indicated (Watts, 1966).

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The Significance of Anions in the Protection by Substrates of Adenosine Triphosphate-Creatine Phosphotransferase Against Inhibition by Iodoacetamide

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Rabbit muscle creatine kinase was first observed to be protected against inhibition by iodoacetamide by an equilibrium mixture of substrates (Watts & Rabin, 1962). In these experiments the ionic strength was controlled with NO_3^- ions. The enzyme was also found to be strongly protected by creatine plus Mg-ADP in the presence of 50mm- Cl^- ion (O'Sullivan, Diefenbach & Cohn, 1966). These univalent anions inhibit normal enzyme activity to varying degrees, but acetate does not. This indicated the desirability of determining the precise effect of anions in iodoacetamide experiments.

By using 50mm-bicine-NaOH buffer throughout it was found that creatine (40mm) plus ADP (1mm) and magnesium acetate (10mm) were completely without effect on the inhibition of creatine kinase by iodoacetamide at various pH values. However, the addition of 0.1M-sodium chloride resulted in the previously observed protection. Nitrate was an even better protecting agent and the order of efficiency of a series of anions in facilitating protection was $\text{NO}_3^- > \text{Cl}^- > \text{HCO}_3^- > \text{Br}^-$; acetate ions were completely without effect. The anions without substrates did not affect the rate of inhibition and the type of univalent cation present was unimportant.

Lineweaver-Burk plots with Mg-ATP as the variable substrate and fixed concentrations of Mg-ADP as the competitive inhibitor gave K_i values for the Mg-ADP complex of 0.24mm in 0.1M-acetate and of 0.055mm in 0.1M-chloride. With phosphocreatine as variable substrate K_i for creatine was 40mm in acetate but 6mm in chloride. However, the K_m values for creatine and Mg-ADP acting as substrates were not decreased in chloride compared with acetate. It would appear that the inhibitory anions selectively mediate the binding of creatine and Mg-ADP to the enzyme to form a dead-end complex that protects the essential thiol group. The consequence of this complex is also seen in normal enzyme progress curves in which the initial part becomes more curved as a more inhibitory anion is used, rendering kinetic data unreliable.

With acetate as added anion an equilibrium mixture of substrates gave a small but reproducible protection against iodoacetamide. This could represent a short-lived intermediate complex forming part of the mechanism suggested by Watts & Rabin (1962). Sterically suitable anions may