carbonic anhydrase III with an estimated purity of greater than 95%, as judged by electrophoresis in sodium dodecyl sulphate/polyacrylamide gels.

Ion-exchange chromatography and salt fractionation were used to purify the bovine carbonic anhydrase III. Bovine muscle was homogenized and adjusted to 40% saturation with (NH₄)₂SO₄. The supernatant was applied to a DEAE-cellulose column (2.5 cm × 25 cm) at pH 8.7 in 0.01 M-Tris and elution was with the same buffer. The enzyme was then applied to a CM-cellulose column (1.5 cm × 110 cm) in 0.01 M-sodium phosphate, pH 6.6. The active fractions were eluted with a linear gradient of NaCl (150 ml, 0.3 M-0.2 M-NaCl, in 0.1 M-sodium phosphate, pH 6.6). The resulting preparation was pure, as judged by electrophoresis in sodium dodecyl sulphate/polyacrylamide gels. (Both human and bovine carbonic anhydrase III had mol. wts. of 27000–28000.)

The kinetic properties of these two carbonic anhydrase III isoenzymes were similar, but in sharp contrast with those of carbonic anhydrase I and II, thus the human carbonic anhydrase III gave 5.0% of the CO₂ hydration rate of isoenzyme CAI under identical assay conditions and about 1% of the esterase activity of carbonic anhydrase I (2.5 nmol/min per mg of enzyme) measured with p-nitrophenyl acetate as substrate (Tashian, 1969). The bovine carbonic anhydrase III gave about 30% of the CO₂ hydrase activity of carbonic anhydrase I and the esterase activity was undetectable under conditions similar to those described for the human enzyme.

Inhibition kinetics with acetazolamide showed that the bovine carbonic anhydrase III was only weakly inhibited in comparison with isoenzymes CAI and CAII. For example, the inhibition constant (I₅₀; M⁻¹) for bovine isoenzyme CAI was 2.0 × 10⁻³, and 1.1 × 10⁻⁷ for isoenzyme CAII, whereas for bovine isoenzyme CAIII the value was 2400 × 10⁻⁷. Determinations of energy of activation gave values of 1029 and 714 kJ/mol for bovine isoenzymes CAI and CAI respectively and 395 kJ/mol for isoenzyme CAIII.

These results indicate that isoenzyme CAIII, which is predominant in red skeletal muscle, has unique properties. It has very low CO₂ hydrase and esterolytic activity relative to the other carbonic anhydrase isoenzymes and is poorly inhibited by sulphonamides. However, the CAIII enzyme protein is present at a concentration of about 1 mg/g wet weight of muscle, and the true catalytic function of the enzyme remains a paradox. Preliminary sequence analyses (R. E. Tashian, unpublished work) show a number of regions of homology for CAI, CAII and CAIII confirming the common evolutionary origin of these isoenzymes.

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Inhibition of Rabbit Muscle Creatine Kinase by Iodomethane

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Creatine kinase from all sources so far examined contains one highly reactive thiol group per subunit, the alkylation of which results in either a total loss or a very considerable loss of enzyme activity. This thiol group is commonly known as the 'essential' thiol group (Watts, 1973). Hooton (1968) reported that reaction of chicken brain-type creatine kinase with iodomethane, under conditions that were claimed to fully alkylate the reac-
Fig. 1. Progress curves comparing the inhibition of rabbit muscle creatine kinase by different alkylating agents at pH 8.6 and 30°C

The inhibitors used were: •, iodomethane (19.4 mM); ○, iodoacetate (1 mM); △, iodoacetamide (0.2 mM).

tive thiol group, resulted in a residual enzyme activity of 25–30% of the initial activity. More recently Kenyon and his co-workers (Smith & Kenyon, 1974; Smith et al., 1975) reported that reaction of the rabbit muscle enzyme with S-methyl methanethiosulfonate, under conditions where each thiol group per subunit was fully modified, again resulted in a residual activity, this time of about 20% of the unmodified value. An explanation was advanced by both groups of workers that residual activity was a feature of the use of a blocking agent that is smaller than the -CH,COO- and -CH2CONHZ groups commonly used hitherto.

Introduction of a methyl group by use of iodomethane has never been reported for rabbit muscle creatine kinase and, on the above evidence, alkylation might be expected to be accompanied by a significant amount of residual activity. We have now carried out this experiment and do not find the expected result.

Creatine kinase was prepared from rabbit muscle by method B of Kuby et al. (1954) and fully reduced by dialysis against dithiothreitol.

Alkylation was carried out by equilibrating 4 vol. of enzyme (58.75 µg of protein/ml) in 0.1 M N,N-bis-(2-hydroxyethyl)glycine/NaOH buffer, pH 8.6, at 30°C for 5 min and then adding 1 vol. of the inhibitor to give a final concentration as shown in the legend to Fig. 1. At appropriate time intervals samples (0.1 ml) were transferred to 0.5 ml of cold cysteine solution in the same buffer to stop the reaction. The cysteine concentration was 5 mM for iodoacetate and iodoacetamide, and 20 mM for iodomethane. Residual enzyme activity was determined in the direction of phosphocreatine synthesis by using the phosphate assay method at 30°C as described by Watts & Moreland (1970), but with magnesium acetate rather than with MgSO4 and with an incubation time of 10 min. Rate of product formation was essentially linear with time over this period.

Fig. 1 compares the efficacy of iodomethane as an inhibitory agent with iodoacetate and iodoacetamide. Under these conditions <3% of enzyme activity remained with any of the inhibitors after incubation for 30 min. Iodomethane was in no way different as an alkylating agent from either iodoacetate or iodoacetamide.

It may be concluded that whatever the explanation for the residual activity observed by Kenyon and his co-workers after attachment of the CH3S— group to the essential thiol groups of rabbit muscle creatine kinase the answer does not reside in the small size of the agent used.

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The availability of insulins chemically modified at specific sites on the molecule has contributed greatly to the understanding of the relationships between the structure and biological activity of the hormone (Freychet et al., 1974; Gliemann & Gammeltoft, 1974). The metabolism of insulin, proinsulin and ten semi-synthetic analogues of insulin has been investigated in an attempt to determine the nature of the site, or sites, on the insulin molecule involved in this process.

The insulin analogues studied were chemically modified at the free amino groups of the glycine-A1, phenylalanine-B1 or lysine-B29 residues or included a synthetic cross-link between residues A1 and B29. The cross-linked insulins were studied in view of their similarity to proinsulin (Brandenburg et al., 1973). Degradation of insulin, proinsulin and the chemically modified insulins, both in vivo and in vitro, was measured by loss of immunoreactivity (Thomas et al., 1975).

Studies in vivo were conducted in anaesthetized greyhounds that had been starved overnight. By using a priming-dose infusion technique (Jones et al., 1976), the metabolic clearance rate and half-life (\( t_1/2 \)) of insulin, proinsulin and the chemically modified insulins were determined. The metabolic clearance rate for insulin was 17.4 ml/min per kg body wt. at a serum concentration of insulin of 1 nM and the \( t_1/2 \) was 4.6 min. Proinsulin and insulins modified at residues A1 and B29, including the A1–B29-cross-linked insulins, had markedly lower metabolic clearance rates (4.5–8.8 ml/min per kg) and the \( t_1/2 \) was correspondingly prolonged (10.6–13.5 min). Insulins modified at residue B1 with clearance rates of 12.25–16.2 ml/min per kg did not differ significantly from insulin.

The metabolic clearance rate of insulin depends on a number of factors, including the rate of excretion, the presence and activity of degrading enzymes and access to the sites of degradation. There is evidence (Varandani et al., 1972) that the enzymic degradation of insulin by rat liver occurs in a stepwise manner: first, a cleavage of insulin at the disulphide bonds by GSH–insulin transhydrogenase (glutathione–protein disulphide oxidoreductase, EC 1.8.4.2), and, secondly, hydrolysis of the resulting A and B polypeptide chains to low-molecular-weight compounds. In addition, it has been suggested that GSH–insulin transhydrogenase may be the rate-controlling enzyme in the degradation of insulin by the liver.

The rates of degradation of insulin, proinsulin and a number of chemically-modified insulins catalysed by a purified preparation of GSH–insulin transhydrogenase were determined. The enzyme was prepared from the microsomal fraction of rat liver as

§ Abbreviation: GSH, reduced glutathione.