contained 69% of the applied thiol. Some RNA and a discrete peak of low-molecular-weight thiol compounds were retained in the gel. The thiol-containing protein peak was further fractionated on microgranular DEAE-cellulose giving seven components. About 40% passed through the column unabsorbed and, apart from 8M-urea, this protein could only be dissolved in 1M-acetic acid. When the latter solution was passed through a CM-cellulose column, quantitative absorption occurred. The main bulk of the thiol material could be eluted with 20mM-HCl, giving a fraction containing 78nmoles of SH/mg. of protein. Analysis gave a ratio of Asp + Glu : Lys + Arg + His of 2:1.

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Effects of Trypan Blue on Rat Liver Heterolysosomes

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The effect of intralysosomal trypan blue on the digestive capacity of rat liver heterolysosomes has been studied, the experimental methods being based on those described by Mego, Bertini & McQueen (1967). Bovine serum albumin was labelled with ^{125}I by the method of McConaghey & Dixon (1966) and denatured as described by Mego *et al.* (1967). Male Wistar rats (275–310g.) were injected intravenously with 1·4mg. of protein/kg. body wt. and killed, and their livers were perfused with cold 0·9% NaCl. After homogenization in cold 0·25M-sucrose a pellet sedimenting between 11000*g*-min. and 330000*g*-min. was prepared by centrifugation. This pellet was suspended in tris–acetic acid buffer, pH 7·4, containing 0·25M-sucrose and incubated at 37°, and samples were withdrawn at intervals up to 2hr. Each sample was assayed for trichloroacetic acid-soluble radioactivity (a measure of the extent of proteolysis) and for non-sedimentable trichloroacetic acid-insoluble radioactivity (a measure of free albumin and hence of heterolysosome rupture).

Acid-soluble radioactivity increased from 10% to 36–41% of the total radioactivity over 2hr.; no increase was observed when 0·2% of Triton X-100 was included in the incubate, indicating that proteolysis occurs within osmotically active particles. Free albumin increased from 4% to 25–35% in the first 30min. and remained at this value for the duration of the incubation. Inclusion of 0·2% of Triton X-100 in the incubate caused the immediate release of 80–90% of the albumin.

Rats that had received trypan blue (75mg./kg.) subcutaneously 24hr. before injection with albumin consistently showed a lower rate of protein breakdown than controls, trichloroacetic acid-soluble radioactivity reaching only 20% after 2hr. This decreased rate of proteolysis was accompanied by an increased release of free albumin, which reached 50% of the total radioactivity within 30min. This effect of intralysosomal trypan blue on heterolysosome stability probably explains the lower rate of protein breakdown observed in incubates of particles from dye-treated rats. However, since trypan blue inhibits lysosomal enzymes (Lloyd, Beck, Griffiths & Parry, 1968), intralysosomal enzyme inhibition may also contribute to the decreased rate of protein breakdown.

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Pyruvate Carboxylase in Lactating Rat and Rabbit Mammary Gland

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Though the role of pyruvate carboxylase (EC 6.4.1.1) in gluconeogenesis is well established, it has been postulated (Wise & Ball, 1964; Ballard & Hanson, 1967) that in adipose and liver the enzyme has a role in lipogenesis. Lactating mammary gland has a high lipogenic activity (Fritz, 1961; Masoro, 1962), yet the presence of pyruvate carboxylase has not been reported in this tissue. We have therefore investigated some properties of this enzyme in lactating rat and rabbit mammary gland.

Preliminary experiments indicated that pyruvate carboxylase was present in both the mitochondrial and particle-free fractions from homogenates of rat mammary gland, but confined to the mitochondrial fraction of homogenate of rabbit mammary gland. Optimum conditions for the assay of this enzyme in these fractions have been determined. The cofactor requirements do not differ significantly

from those reported for the enzyme in avian liver (Utter & Keech, 1963; Keech & Utter, 1963) and rat adipose (Ballard & Hanson, 1967), though the pH optimum (8·5 for the enzyme in rat mammary fractions and 7·8 for the rabbit mammary-gland enzyme) was somewhat different. Albumin (fat-free) produced a 3-fold increase in the specific activity of the enzyme in the soluble fraction of rat mammary gland. In all cases pyruvate carboxylase showed a dependence on added acetyl-CoA.

Under optimum assay conditions, cell-fractionation studies in both 0·3M- and 0·88M-sucrose as homogenization medium and using marker enzymes confirmed the subcellular distribution of pyruvate carboxylase in these tissues. Evidence will be presented that the soluble enzyme is not an artifact due to mitochondrial damage, nor is mitochondrial pyruvate carboxylase due to an adsorption of the enzyme from the soluble fraction.

Avidin inhibited the enzyme in the mitochondrial fraction of both tissues, though the inhibition was incomplete. In contrast, the soluble enzyme from rat mammary gland was completely inhibited by avidin. The specific activity of the mitochondrial enzyme from rat mammary gland increased when the fraction was subjected to freeze-drying (2·3-fold), freezing-and-thawing (approx. 5-fold) or ultrasonic treatment (3·6-fold). The mitochondrial enzyme from rabbit mammary gland behaved differently, since little or no change in specific activity was observed under these conditions.

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The Chemical Nature of the Site of Action of Dicyclohexylcarbodi-imide in Mitochondria

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Dicyclohexylcarbodi-imide (DCCD) is a potent inhibitor of oxidative phosphorylation (Beechey, Holloway, Knight & Roberton, 1966; Beechey, Roberton, Holloway & Knight, 1967). Evidence has been adduced that a covalent bond is formed between the di-imide moiety and the site of action. Here we present evidence that proteolipid (Folch & Lees, 1951) is the site of action of DCCD.

Ox heart mitochondria that had been incubated overnight at 0° with 1 μmole of [^{14}C]DCCD/g. of protein contained, on reisolation, 0·9–1·0 μmole of DCCD/g. of protein. Extraction of these mitochondria with organic solvents (Folch, Lees & Sloane-Stanley, 1957) removed an average of 75% of the radioactivity, which, when analysed by thin-layer, paper, ion-exchange and adsorption chromatography and by gel filtration became irreversibly adsorbed on the support material and thus was neither DCCD nor *NN'*-dicyclohexylurea.

Phospholipid was removed from these extracts by dialysis against the sequence of solvents described by Tenenbaum & Folch-Pi (1966). Daily, the dialysate was concentrated, analysed by thin-layer chromatography and the ^{14}C content measured. 15% of the radioactivity was extracted in the first 2 days of dialysis. This proved to be mainly *NN'*-dicyclohexylurea. A further 15%, most of which was chromatographically similar to the dialysed extract, was removed over 25 days, its release correlated with the aging of the dialysis membrane. The protein contents of the dialysis sac, which became, after drying, insoluble in all but phenol–acetic acid–water (2:1:1, w/v/v), showed two radioactive spots on thin-layer chromatography.

A 350ml. portion of a similar mitochondrial extract was layered overnight under 5l. of water and the accumulated interfacial and chloroform-soluble materials were isolated. They contained 81 and 11·5mg. of protein respectively. Acetone and diethyl ether extractions of these fractions removed much lipid material. However, the greater proportion of the counts remained in the extracted material. Solution of these materials in the phenol–acetic acid–water systems and analysis by thin-layer chromatography suggested that these 'interfacial' and 'chloroform-soluble' protein fractions corresponded to the two radioactive materials found in the sac. Treatment of the chloroform-soluble fraction with Pronase solubilized 25% of the radioactivity, and this soluble fraction ran as a single material on thin-layer chromatography and was eluted as a single peak from Sephadex G-10.

We conclude that DCCD combines with at least two proteolipid fractions in mitochondria. The adherence of radioactivity to the proteolipid fractions throughout these varied physical and chemical procedures supports the theory that a covalent bond is formed between proteolipid and the di-imide moiety.

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