Diurnal Variations in Rat Liver 3-Hydroxy-3-methylglutaryl-Coenzyme A Reductase Activity in Relation to Feeding

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The 3-hydroxy-3-methylglutaryl-CoA reductase (EC 1.1.1.34) activity of rat liver microsomal fraction varies diurnally: in rats allowed to feed at will and subjected to alternate 12h periods of light and darkness the activity is maximal during the dark period and minimal during the light period (Hamprecht *et al.*, 1969; Shapiro & Rodwell, 1971; Higgins *et al.*, 1971). The present communication describes daily changes in this enzyme activity in rats trained to feed during the daytime; such a study seemed relevant in view of the known tendency of rats to feed during the dark (Suttie, 1968).

Experimental

Female Wistar rats were purchased from the Manston Research Centre, Margate, Kent, U.K., and weighed 90g. They were kept in a room artificially illuminated between 09:00h and 21:00h, and in darkness between 21:00h and 09:00h. Control animals were allowed continuous access to food, but test rats were fed from 09:00h to 12:00h each day. (All times quoted here refer to British Summer Time, which is 1h ahead of Greenwich Mean Time.) Water was accessible at all times. Animals were maintained for 10 days before the experiment, and, beginning at 24:00h of day 10, measurements of enzyme activity were made; at 09:00h some of the test rats were fed as usual but others were given no food.

Liver microsomal fraction was prepared as described by Tata (1969). Enzyme activity was measured by incubating microsomal preparations with 3-hydroxy-3-methyl[3-¹⁴C]glutaryl-CoA (specific radioactivity 0.60 μ Ci/ μ mol) and a system for generating NADPH, and subsequently isolating and counting the radioactivity of the product, mevalonate. The substrate was prepared chemically in two stages. First, 3-hydroxy-3-methyl[3-¹⁴C]glutaric acid (NEN Chemicals G.m.b.H., Frankfurt/Main, Germany) was converted into the cyclic anhydride by the method of Goldfarb & Pitot (1971). Secondly, the anhydride was coupled to CoA (Boehringer Corp., London W.5, U.K.) under the conditions described by Stegink & Coon (1968). Incubations for enzyme assay were done in duplicate at 37°C for 30min, and the mixture, of total volume 0.25ml,

was as described by Shapiro & Rodwell (1971) except that the concentration of microsomal protein was not allowed to exceed 4mg/ml. Experiments showed that when higher concentrations were used the enzyme activity ceased to be proportional to the quantity of protein used. Similarly enzyme activity was not linear with time when incubations were continued for longer than 30min. These conditions were observed to apply to maximally and minimally active preparations alike. After the incubation mevalonate was extracted from the reaction mixture, which contained 1.3mg of unlabelled 'carrier' mevalonate, and isolated by t.l.c. on silica gel G as described by Shapiro *et al.* (1969). Chromatograms were scanned, with a Panax Thin-Layer Scanner, to locate the radioactive mevalonate, and the appropriate area was cut out and its radioactivity counted with a Nuclear-Chicago Liquid Scintillation Spectrometer and a scintillation fluid consisting of 2-ethoxyethanol (300ml), 1,4-dioxan (300ml), 2,5-diphenyloxazole (4g) and naphthalene (80g), made up to 1 litre with A.R. toluene. Counts were corrected for background and counting efficiency, and the amount of mevalonate formed was calculated from the specific radioactivity of the substrate. The extraction of mevalonate is incomplete; recovery was checked repeatedly by extracting known amounts of [2-¹⁴C]mevalonate from simulated reaction mixtures and was 45–50%. A suitable correction was made when the enzyme activity was calculated.

One unit of enzyme activity forms 1nmol of mevalonate in 1min. Specific enzyme activity is expressed as units/mg of microsomal protein. Protein was measured by the method of Lowry *et al.* (1951), with dry bovine serum albumin (type F; Sigma Chemical Co., St. Louis, Mo., U.S.A.) as standard protein.

Results and discussion

Rats are reluctant to feed during the day, and the test animals do not gain weight during the first 2–3 days after subjection to restricted access to food. Thereafter each rat gains about 4g daily, showing that the animals are accustomed to their new conditions. The diurnal changes in 3-hydroxy-3-methylglutaryl-CoA reductase activity observed (Fig. 1) in control

Fig. 1. Diurnal variations in rat liver 3-hydroxy-3-methylglutaryl-CoA reductase activity in relation to feeding

Control rats (○) were allowed continuous access to food. Test rats (●) were trained, as described in the text under 'Experimental', to feed from 09:00h to 12:00h, as indicated in the figure by the horizontal bar; ■ denotes test rats fed as usual on the day of the experiment; ▲ denotes test rats not given food on the day of the experiment. Each point is the mean of determinations on three to 17 rats. Vertical lines indicate $\pm$ S.E.M. Differences between means were evaluated where appropriate by applying a *t* test; values of *P* are given in the text. Units of enzyme activity are defined in the text under 'Experimental'.

rats are in complete agreement with those described by others (Hamprecht *et al.*, 1969; Shapiro & Rodwell, 1971; Higgins *et al.*, 1971). In the test animals the pattern is virtually reversed, this enzyme activity now being at its height during the light period. The peak of activity is therefore related to the time of feeding and does not require the onset of darkness, although the juxtaposition of light and dark periods undoubtedly determines when the rat will feed if allowed free choice (Huber & Hamprecht, 1972).

The response to feeding is twofold. In the test rats the enzyme activity is higher at 09:00h than at 24:00h ($P < 0.05$); it therefore rises before feeding begins, resulting in a peak of activity even in the rats denied food on the day of the experiment. This rise must be due to the fact that the rats are accustomed to feed at 09:00h; during the same period activity in control animals, which were observed to consume practically no food between 09:00h and 12:00h, falls rapidly. Food consumption is nevertheless not without effect; in the test rats fed as usual on the day of the experiment the enzyme activity continues to rise on feeding and at 12:00h is higher than at 09:00h ($P < 0.001$). During the next 6h it remains high, whereas in the test animals denied food it falls. Thus at 15:00h and 18:00h the enzyme activity in the fed rats is higher ($P < 0.001$) than in those not fed.

Evidently two stimuli are responsible for the rising phase of this diurnal variation. The first begins to act in anticipation of feeding and at a time determined by the time at which the rat habitually feeds. There is no evidence to suggest the nature of this stimulus. The second is the consumption of food, although not necessarily the absorption of a nutrient from the diet.

Two previously opposing views may now be resolved. Hamprecht *et al.* (1969) observed a nocturnal peak of this enzyme activity in rats starved for 24h and concluded that food consumption is not a factor in the diurnal variation. Our results do not support such a conclusion, but suggest the alternative explanation that the first stimulus persists during starvation and acts when the rats were previously accustomed to feeding. Since the rats had previously been fed *ad libitum*, this would be during the night. On the other hand, the experiments of Edwards *et al.* (1972) permitted the observation of changes following, but not preceding, feeding, and led to the incomplete view that food intake is mainly responsible for the diurnal variation. It should be noted that the incorporation of intravenously injected acetate into liver non-saponifiable lipid, and not the enzyme activity directly, was measured in the latter study.

Huber *et al.* (1972) have described a nocturnal peak of this enzyme activity in adrenalectomized rats. Their conclusion, that adrenal hormones play no role in the diurnal variation, may not be correct if food consumption alone is able to evoke such a response and if adrenalectomy does not cause the rat to change its normal feeding habits.

Higgins *et al.* (1971) suggest that the rising phase of this enzyme activity in rats fed *ad libitum* reflects an increased rate of enzyme synthesis, and their evidence is consistent with this view. It now seems necessary to ascertain whether this observation applies to one or both of the stimuli concerned.

3-Hydroxy-3-methylglutaryl-CoA reductase is

widely considered to be important in controlling the rate of cholesterol biosynthesis. The rate of incorporation of acetate into cholesterol does follow a diurnal rhythm similar to that of the enzyme activity (Back *et al.*, 1969; Horton *et al.*, 1970; Edwards *et al.*, 1972). Beyond this, speculation on the significance of the phenomena described here would be premature.

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