A fraction derived from brewer's yeast inhibits cholesterol synthesis by rat liver preparations in vitro

BY E. S. HOLDSWORTH, D. V. KAUFMAN AND E. NEVILLE

Biochemistry Department, University of Tasmania, Hobart, Tasmania, Australia

(*Received 21 February 1990 – Accepted 3 August 1990*)

Brewer's yeast was grown on a defined medium containing tracer ^{51}Cr with or without added chromium. The two batches of yeast contained 10 μg/g (high-Cr) or 80 ng/g (low-Cr). Extracts were prepared and fractionated. A third batch of yeast (third batch) was grown with added Cr, and fractionated. Rats were reared on either rat cubes (normal diet) or on a low-Cr diet (low-Cr), or on rat cubes with added cholestyramine (cholestyramine diet). Preparations of rat liver, both cell-free and intact hepatocytes, incorporated acetate-carbon into fatty acids and cholesterol. These processes were inhibited by a yeast fraction containing small, neutral, water-soluble compounds. The degree of inhibition was the same whether the liver came from normal rats or rats fed on the low-Cr diet. Similarly the inhibitory effect was found with identical amounts of extracts from low- or high-Cr yeasts. Therefore, Cr compounds do not appear to account for the inhibitory effects of brewer's yeast. Use of other substrates indicated that the site of inhibition of sterol synthesis was apparently between acetyl-CoA and mevalonate. One inhibitory substance was isolated from yeast and was found to be nicotinamide riboside. This may have been produced from NAD(P) during the preparation of yeast extracts, and it may be produced from dietary yeast supplements during digestion in vivo. Nicotinamide riboside may be partly responsible for the reported effects of yeast supplements on plasma lipids in humans.

Brewer's yeast: Chromium: Cholesterol biosynthesis: Rat

High serum cholesterol and low serum high-density-liprotein (HDL)-cholesterol are risk factors for coronary artery disease (CAD) (Miller *et al.* 1977). During investigations to study the effects of brewer's yeast on the prevention of glucose intolerance in humans, it was found that dietary supplements of brewer's yeast lowered serum cholesterol or raised serum HDL-cholesterol, or both (Offenbacher & Pi-Sunyer, 1980; Elwood *et al.* 1982; Grant & McMullen, 1982; Vinson & Bose, 1984), although other studies have failed to demonstrate such effects (Rabinowitz *et al.* 1983; Hunt *et al.* 1985; Offenbacher *et al.* 1985). Brewer's yeast has been considered to be a good source of the organically-bound chromium alleged to be the substance involved (reviewed by Wallach, 1985), and animals reared on low-Cr diets have been reported to have raised serum cholesterol levels (Schroeder, 1969; Stoecker & Oladut, 1985). Supplementation of the diet with inorganic Cr has been reported to lower serum cholesterol or raise HDL-cholesterol, or both, in humans (Riales & Albrink, 1981) and animals (Schroeder, 1969; Stoecker & Oladut, 1985) and to decrease aortic plaques in rabbits (Abraham *et al.* 1982), but again other studies have failed to show an effect on serum lipids (Preston *et al.* 1976; Rabinowitz *et al.* 1983; Uusitupa *et al.* 1983; Offenbacher *et al.* 1985; Potter *et al.* 1985; Bourn *et al.* 1986; Li & Stoecker, 1986). Donaldson *et al.* (1985) were unable to attribute differences in plasma cholesterol entirely to differences in dietary Cr levels. Nevertheless serum or plasma Cr levels have been found to be substantially lower in patients with CAD than in patients with other types of heart disease (Newman *et al.* 1978; Simonoff *et al.* 1984; Conri *et al.* 1986).

In view of the importance of CAD, it seemed desirable to test brewer's yeast fractions

for effects on cholesterol homeostasis, with the aim of identifying any active component and assessing the involvement of Cr. The liver is the major site of cholesterol synthesis, and some inhibitors of cholesterol synthesis have been found to improve blood lipid profiles in experimental animals (for review, see Brown & Goldstein, 1986). Therefore, inhibition of liver cholesterol synthesis is one possible way in which yeast supplements might affect cholesterol levels, although inhibition of liver cholesterol synthesis does not necessarily lower blood cholesterol levels (Brown & Goldstein, 1986). The present paper reports on effects of brewer's yeast fractions on cholesterol synthesis by rat liver preparations in vitro. Normal rats, and also rats raised on a low-Cr diet or on a cholestyramine diet, were used in the present work; yeast fractions were derived from yeast preparations differing more than 100-fold in Cr content.

EXPERIMENTAL METHODS

Growth and fractionation of brewer's yeast

Brewer's yeast was grown on a defined medium of glucose, ammonium sulphate, vitamins and minerals as described by Davies *et al.* (1985). Two batches of yeast, one grown with trace levels of Cr salt and another with 1 mg Cr^{3+}/l were extracted with 0·5 M-ammonia and fractionated on Biogel P_4, DEAE Sephadex A25 at pH 4, and Dowex 50 in H^+ form as previously described (Davies *et al.* 1985; Holdsworth & Neville, 1988). Fraction N1 contained water-soluble material, with molecular weight less than 1300, which did not bind to Dowex 50 H^+. A third batch of yeast was grown in medium containing 1 mg Cr^{3+}/l, harvested and fractionated as shown in Fig. 1. After extraction with 0·5 M-ammonia the extract was passed through a membrane filter passing material of less than 10000 molecular weight and freeze-dried. A solution of the residue in water at pH 8·5 was passed down a column (50 × 1000 mm) of 150 g DEAE Sephadex A25 which had been equilibrated with 0·05 M-ammonia solution. With these conditions inhibitory material was retarded on the column. The column was washed with 250 ml 0·05 M-ammonia, then with 700 ml distilled water followed by dilute acetic acid (0·1 M) and finally with 1 M-acetic acid. Fractions were freeze-dried and portions tested for their ability to inhibit incorporation of [1-^{14}C]acetate into non-saponifiable lipid. Fractions DE2, DE3 and DE4 had this activity and each was fractionated further by passing through a column of Dowex 50. With fractions DE2 and DE3 the column of Dowex 50 was in H^+ form and it was subsequently found that the low pH encountered within the column destroyed some of the inhibitory activity. For this reason fraction DE4 was fractionated on a column of Dowex 50 in NH_4^+ form at pH (water effluent) 4·0. In all instances the inhibitory activity was not retained by the Dowex 50, therefore material washed through these columns with water was collected and freeze-dried to give fractions NA, NB, NC, ND.

Fraction ND was further fractionated by high-performance liquid chromatography on a Pharmacia column of polyanion (S1 HR 5/5) which had been well-washed with 1 M-acetic acid and then water. A total of five 1 ml portions were processed using a linear gradient, water to 0·1 M-acetic acid, with a flow-rate of 1 ml/min. Fractions were selected on the basis of absorbance at 254 nm, and the material collected from each peak shown in Fig. 2 was collected and freeze-dried. Seven fractions were obtained (ND1–ND7).

Yeast fractions were examined by thin-layer chromatography in several systems: Polyamide F_{254} plates (Merck) with sodium acetate in water (100 g/l) as solvent; Kieselgel F_{254} plates (Merck) and Kieselgel plates without fluorescent indicator using *n*-propanol–water–concentrated ammonia (66:33:1, by vol). Detection of compounds was by quenching of the fluorescent indicator, by staining with iodine vapour, by staining with ninhydrin and with naphthoresorcinol (Smith, 1969).

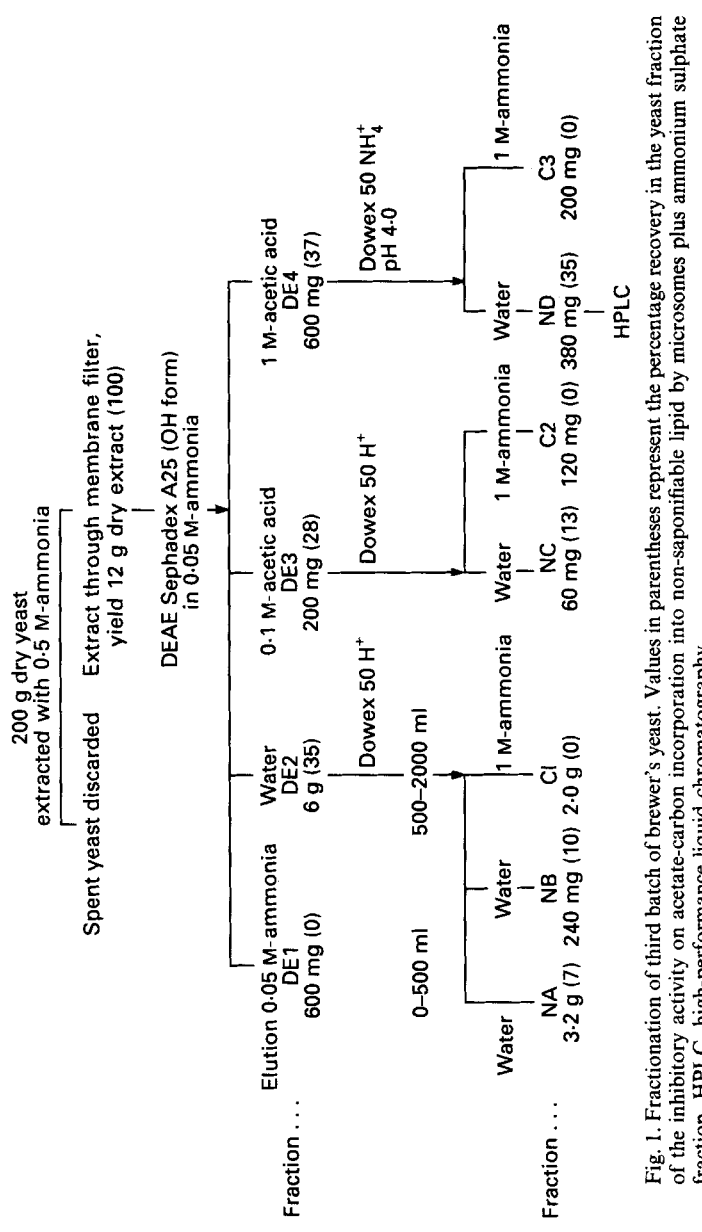

Fig. 1. Fractionation of third batch of brewer's yeast. Values in parentheses represent the percentage recovery in the yeast fraction of the inhibitory activity on acetate-carbon incorporation into non-saponifiable lipid by microsomes plus ammonium sulphate fraction. HPLC, high-performance liquid chromatography.

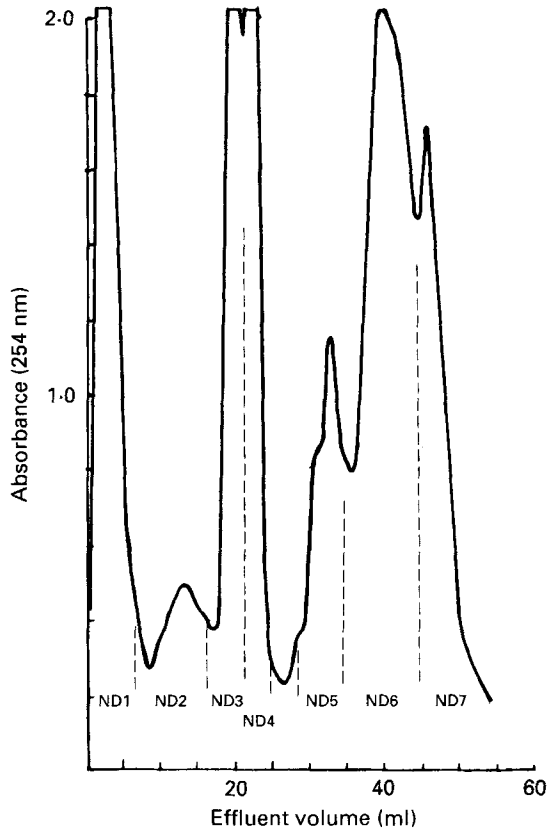

Fig. 2. High-performance liquid chromatography (HPLC) of fraction ND was performed as described on p. 286. Absorbance at 254 nm of effluent is shown. (—), The effluent was cut to give the seven fractions ND1–ND7.

Treatment of NADH and NADPH

Portions (10 mg) of each of the reduced nicotinamide nucleotides (Sigma Chemical Co, St Louis, USA) were dissolved in 200 ml 0·5 M-ammonia solution and kept at 20° for 2 h and then freeze-dried. The material was then dissolved in 100 ml 1 M-acetic acid and kept at 20° for 2 h and freeze-dried. The solids were dissolved in 5 ml distilled water and stored at −15°.

Animals and diets

Normal diet. Hooded Wistar male rats were raised from weaning on commercial rat cubes and water *ad lib.*, at a minimum temperature of 20–22 °C, and a minimum relative humidity of 40–45%. Lighting was from 06.00 to 18.00 hours. Rats were used when they were approximately 200 g weight and were killed at approximately 09.00 hours.

Cholestyramine diet. Six rats were fed on crushed rat cubes mixed with cholestyramine, 30 g/kg, for the final 7–10 d before preparation of 10000 ***g*** supernatant fraction (S_{10} fraction) or microsomes in order to maximize cholesterol synthesis. These rats were killed at midnight, instead of the usual 09.00 hours, in order to have the hydroxymethylglutaryl (HMG)-CoA reductase (*EC* 1.1.1.34) activity near its diurnal maximum.

Low-Cr diet. Rats were raised from weaning (about 50 g) on the low-Cr diet supplied by Teklad (Wisconsin, USA) which contained 300 g Torula yeast/kg, 600 g sucrose/kg, lard, vitamins, minerals and amino acids (Teklad 84485), and which contained less than 0·1 μg

Cr/g (Holdsworth & Neville, 1990). To minimize contamination by Cr the rats were housed in plastic cages with raised aluminium-mesh floors and given deionized water to drink from all-glass containers. Room air was not filtered. Lighting was from 06.00 to 18.00 hours. The rats were used when they were approximately 200 g weight, after 7–8 weeks on the diet. Rats which had been reared on this diet were probably only marginally deficient in Cr (Schroeder, 1969), but adipocytes from rats reared in this way responded to yeast extracts (Davies *et al.* 1985) in the manner reported for Cr-complexes.

Isolation and incubation of hepatocytes, extraction of lipids

Hepatocytes were isolated from rats which had been fed on a normal diet, and were incubated as previously described (Holdsworth & Neville, 1988). Incubation flasks contained 20 mg dry weight of cells plus 20 mM-glucose plus radioactive metabolites as shown in Table 1 (p. 292) in a total volume of 4 ml. When added [1-^{14}C]acetate was 1 mM, 3×10^5 disintegrations/min (dpm) per ml; [2-^{3}H]mevalonate was 0·1 mM, $7{\cdot}5 \times 10^5$ dpm per ml; ^3H_2O was 1×10^9 dpm per ml. When added, the yeast fraction was present at 250 μg/ml. Reactions were stopped after 20, 40 and 60 min by addition of perchloric acid ($HClO_4$) to give 0·3 M. Flask contents were centrifuged and the precipitate washed with 0·3 M-$HClO_4$. The drained precipitate was suspended in 2 ml water and 1 ml 10 M-potassium hydroxide and 5 ml ethanol added. Saponification took place at 75° for 90 min. Non-saponified lipid was extracted with light petroleum (b.p. 40–60°), and after acidification of the aqueous layer with concentrated hydrochloric acid the fatty acids were extracted. The extracted lipids were placed in glass scintillation vials, taken to dryness in air and the radioactivity was measured.

Preparation of S_{10} and measurement of fatty acid and sterol synthesis

Rat livers were homogenized and the supernatant fraction (S_{10}) was obtained by centrifugation at 10000 ***g*** for 20 min at 4° as described by Goodwin & Margolis (1978). A 0·8 ml portion of supernatant fraction containing approximately 30 mg protein was pre-incubated at 37° for 20 min, with shaking at 100 rev./min, then 0·2 ml of a mixture of cofactors (Goodwin & Margolis, 1978) plus radioactive metabolites as shown in Table 2 (p. 292) was added, together with 25 μl water or yeast extract. When added [1-^{14}C]acetate was 2 mM, 4×10^6 dpm per ml; [2-^{14}C]mevalonate 0·5 mM, 2×10^5 dpm per ml; DL-[2-^{14}C]HMG-CoA 0·33 mM, 3×10^5 dpm per ml. Incubation was continued for various times up to 10 min at which time the reaction was stopped by addition of ice-cold $HClO_4$ to give 0·6 M. Lipids were extracted from the precipitate and saponified as described previously. Non-saponifiable lipids were extracted and radioactivity was measured as described previously. Ten replicate samples of non-saponifiable lipids were fractionated by gas–liquid chromatography on columns of OV17 (150 g/kg) on Chromosorb G at 270°.

S_{10} preparations incorporated little acetate-C into non-saponifiable lipids unless the S_{10} preparation had been pre-incubated at 37°. As already reported (Goodwin & Margolis, 1978), the approximately 13-fold increase in incorporation which resulted from pre-incubation was presumably due to activation by dephosphorylation of the inactive, phosphorylated form of the rate-limiting enzyme HMG-CoA reductase, since it was abolished by including MgATP in the pre-incubation mixture or by inclusion of fluoride. Pre-incubation had no effect on incorporation of mevalonate.

Preparation of rat liver microsomes and supernatant fraction, and the synthesis of sterols

Livers from rats fed on normal rat cubes (killed at 09.00 hours) or rats fed on the cholestyramine diet (killed at midnight) were used to prepare microsomes and also the fraction precipitating at 40–80 percentage saturation with ammonium sulphate from the

105000 *g* supernatant fraction (ammonium sulphate fraction) by the method of Kuroda & Endo (1977). The preparations were stored in small portions at −80° and thawed as needed for incorporation of precursors into non-saponifiable lipids or for enzyme assays.

Incorporation experiments were performed as described by Kuroda & Endo (1977). Incubation mixtures included microsomes (0·18 mg protein) plus ammonium sulphate fraction (1·7 mg protein) plus cofactors and radioactive substrates in a total volume of 0·2 ml. When added [1-^{14}C]acetate was 1 mM, 9×10^6 dpm per ml; [1-^{14}C]acetyl-CoA was 0·1 mM, $1{\cdot}3 \times 10^6$ dpm per ml; [2-^{14}C]mevalonate was either 0·45 mM, $1{\cdot}35 \times 10^6$ dpm per ml or 35 μM, $4{\cdot}2 \times 10^5$ dpm per ml. Radioactivity was measured in the extracted non-saponifiable lipid. Ten replicate samples of non-saponifiable lipids were fractionated by thin-layer chromatography using the method of Chang *et al.* (1979), and the distribution of ^{14}C on the plates was located with a spark chamber (Birchover Instruments, UK).

Enzyme assays

HMG-CoA synthetase (*EC* 4.1.3.5) and acetoacetyl-CoA thiolase (*EC* 2.3.1.9) activity of the ammonium sulphate fraction were estimated as described by Kurado & Endo (1977).

HMG-CoA reductase was estimated in microsomes obtained in two ways. (1) Microsomes were isolated from S_{10} preparations after incubation of S_{10} at 37° for 20 min to activate the enzyme. The activated S_{10} was centrifuged as described by Ingebritson & Gibson (1981) but at 20° instead of 4°, the microsomes were resuspended and the enzyme assayed as described using NADP plus an NADPH-regenerating system. The HMG-CoA concentration was varied between 330 and 18 μM with appropriate adjustment in incubation time. After acidification, lactonization and thin-layer chromatography, the products were revealed by staining the plates with I_2 vapour and ^{14}C bands were detected with a spark chamber (Birchover Instruments). The [^{14}C]mevalonolactone band was scraped off and counted in ACS II scintillation cocktail with thixotropic gel powder (Packard Instrument Co., USA) and ^{14}C recovered was corrected for recovery of the [^{3}H]mevalonolactone internal standard. (2) HMG-CoA reductase was also assayed in microsomes prepared by the method of Kuroda & Endo (1977) and assayed both at 5 mM-NADPH and at 100 μM-NADPH with appropriate decrease in incubation time.

Acetyl-CoA carboxylase (*EC* 6.4.1.2) was estimated in S_{10} preparations by the method of Holland *et al.* (1984). Glucose-6-phosphate dehydrogenase (*EC* 1.1.1.49) in S_{10} preparations was assayed spectrophotometrically (Lohr & Waller, 1974).

Total cholesterol and HDL-cholesterol

Blood samples were taken at 10.00 hours from the abdominal aorta under light diethyl ether anaesthesia from animals which had been fasted overnight. Samples were allowed to clot for 2 h at 4°. Part of the serum was immediately treated with polethylene glycol 6000 (100 g/l) at pH 10 for 5 min to precipitate low-density lipoproteins and very-low-density lipoproteins, and then centrifuged (Allen *et al.* 1979). The supernatant fraction was used for estimation of HDL-cholesterol, and untreated serum was used for estimation of total cholesterol. Cholesterol was estimated enzymically using a Boehringer Mannheim CHOD-PAP kit, using ALSP serum standard (CSL) and Validate Control.

Miscellaneous

Protein was estimated by the method of Lowry (Layne, 1957). Perirenal and epididymal adipose tissue samples were placed, immediately after removal from the animal, in small air-tight containers and weighed within a few minutes. Radioactivity was measured in ACS II scintillation fluid (Packard Instrument Co., USA) in a Beckman 3801 multi-channel liquid scintillation counter appropriately programmed for quenching and for discrimination

between ^{3}H and ^{14}C. The significance of differences between means was calculated using Student's *t* test.

RESULTS

Effect of yeast fraction N1 on incorporation of precursors into lipids by hepatocytes

Isolated hepatocytes incorporated C from [1-^{14}C]acetate into non-saponifiable and saponifiable lipids, and yeast fraction N1 inhibited these processes equally (Table 1). Results were identical whether fraction N1 was obtained from low- or high-Cr yeast preparations. C from [2-^{14}C]mevalonate was incorporated into non-saponifiable lipid (mean 0·71 (SE 0·01) μmol/h per g dry weight) but at only about one-tenth the rate of incorporation of ^{3}H from [2-^{3}H]mevalonolactone, perhaps due to a permeability phenomenon. Neither label was found in significant amounts in fatty acids. At the concentration used, fraction N1 did not inhibit incorporation of mevalonate or mevalonolactone into non-saponifiable lipid and had only a small effect on incorporation of tritium from tritiated water.

Effect of fraction N1 on incorporation of precursors by S_{10} preparations

Table 2 shows that S_{10} preparations which had been activated by pre-incubation (see p. 289) incorporated acetate-C into non-saponifiable lipids. The non-saponifiable lipids gave only one peak on gas–liquid chromatography, and that peak had the same mobility as cholesterol. Fraction N1, prepared from yeast, inhibited incorporation of acetate-C into non-saponifiable lipids and the degree of inhibition was similar with extracts from high- or low-Cr yeast. The yeast fraction also inhibited the small incorporation into non-saponifiable lipid by non-pre-incubated preparations but had no effect on the degree of activation achieved during pre-incubation. Yeast fraction N1 was a less effective inhibitor of mevalonate incorporation than of acetate incorporation. Results for the expensive substrate HMG-CoA are derived from one experiment in which fraction N1 inhibited HMG-CoA incorporation by 80% while inhibition of acetate incorporation was within the normal range.

The cationic fractions from high- and low-Cr yeast had little inhibitory effect on acetate-C incorporation into non-saponifiable lipid. Saponifiable lipids also were labelled after incubation of [^{14}C]acetate with preparations from animals on normal and low-Cr diets (0·28 (SE 0·02) and 0·32 (SE 0·03) μmol/h per g protein respectively). Fraction N1 from high- and low-Cr yeast inhibited this incorporation somewhat less strongly than incorporation into non-saponifiable lipid; for example 250 μg fraction N1/ml inhibited incorporation into non-saponifiable and saponifiable lipids by 50 and 34% respectively.

Comparison of S_{10} preparations from animals fed on normal and low-Cr diets

S_{10} preparations from animals which had been reared on a low-Cr diet incorporated precursor-C into non-saponifiable lipids more slowly than did preparations from normal animals (Table 2) but the cause of this difference is not known. No difference was found in the glucose-6-phosphate dehydrogenase activity of normal- and low-Cr S_{10} preparations (Table 3), and the activity was much in excess of that needed to supply the NADPH needed for the observed rates of lipid synthesis. Fraction N1 did not inhibit glucose-6-phosphate dehydrogenase under these conditions. HMG-CoA reductase activities in microsomes isolated from S_{10} preparations were similar in normal- and low-Cr preparations (Table 3), and the yields of microsomes from the two preparations were similar. There was no difference between these normal- and low-Cr rats in total serum cholesterol or in HDL-cholesterol (Table 3). There were obvious differences in the amounts of perirenal fat and epididymal fat (Table 3).

Table 1. *Effects of yeast fraction N1* (high-chromium) on incorporation of precursors into lipids by hepatocytes from animals on a normal diet*

(Values given are as means with their standard errors; no. of replicates five)

	Basal incorporation rate into lipids (μmol/h per g dry weight)				Percentage inhibition by fraction N1			
	Non-saponifiable		Saponifiable		Non-saponifiable		Saponifiable	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
^3H_2O	3·7	0·4	16·7	2·3	14	2	−21†	6
[1-^{14}C]acetate	5·0	0·3	7·0	1·1	67	10	63	9
[2-^{3}H]mevalonolactone	7·7	0·4	—	—	−2†	5	—	—

* For details of fractionation procedure, see Fig. 1 and p. 286.
† This represents a stimulation of incorporation.

Table 2. *Effect of yeast fraction N1† from high- or low-chromium yeast on incorporation of precursor-carbon into non-saponifiable lipids by S_{10} preparations‡ from animals fed on normal or low-Cr diets§*

(Values are given as means with their standard errors; no. of replicates six, except for the hydroxymethylglutaryl-CoA (HMG-CoA) values which are based on only two estimates)

Fraction N1 yeast (μg/ml)	Diet	[1-^{14}C]acetate Mean	SE	[2-^{14}C]mevalonate Mean	SE	[2-^{14}C]HMG-CoA
Basal incorporation rate (μmol/h per g protein):						
	Normal	0·84	0·12	5·58	0·90	0·09, 0·13
	Low-Cr	0·31*	0·05	2·80*	0·96	— —
Percentage inhibition by fraction N1:						
Low-Cr						
500	Normal	63	5	23	3	— —
250	Normal	50	4	15	7	83, 81
100	Normal	40	3	0	3	— —
Low-Cr						
500	Low-Cr	53	1	2	3	— —
100	Low-Cr	21	3	—	—	— —
High-Cr						
500	Normal	54	2	—	—	— —
100	Normal	29	3	—	—	— —

Mean values were significantly different from those found on the normal diet: *$P < 0{\cdot}01$.
† For details of fractionation procedure, see Fig. 1 and p. 286.
‡ For details of procedures, see p. 289.
§ For details of dietary treatments, see p. 288 and 289.

Purification and identification of inhibitory material

Thin-layer chromatography of fraction N1 showed eight components by I_2-vapour staining, and six of these showed absorption of u.v. light (254 nm). Attempts to purify the inhibitory material by preparative thin-layer chromatography on Kieselgel using *n*-propanol–water (66:33, v/v) were only partially successful since small amounts of dissolved silica present in zones eluted from the plates were inhibitory in the S_{10} assay system. However two zones, R_F 0·27 and 0·38, showed marked inhibition in the S_{10} assay

Table 3. *Comparison of preparations from rats fed on normal and low-chromium diets*†

(Values are given as means with their standard errors; no. of replicates ten, except for the value for glucose-6-phosphate dehydrogenase (*EC* 1.1.1.49) activity which is based on three replicates, and the K_m which was estimated only once)

	Normal diet		Low-Cr diet	
	Mean	SE	Mean	SE
Glucose-6-phosphate dehydrogenase in S_{10} preparation‡:				
Activity (μmol/min per g protein)	190	20	210	20
Activity in presence of fraction N1 (μmol/min per g protein)	180	20	180	20
K_m (μM)	66	—	68	—
Acetyl-CoA carboxylase (*EC* 6.4.1.2) in S_{10} preparation:				
Activity (μmol/min per g protein)	1·2	0·1	2·1*	0·3
HMG-CoA reductase in S_{10} preparation:				
Activity (μmol/min per g protein)	0·27	0·02	0·32	0·03
Serum cholesterol:				
Total (mM)	1·89	0·05	1·89	0·06
HDL (mM)	1·36	0·05	1·47	0·05
Perirenal fat (g)	1·7	0·1	2·6**	0·2
Epididymal fat (g)	1·4	0·1	1·8*	0·2
Body-weight (g)	209	4	214	9

HMG-CoA, hydroxymethylglutaryl-CoA; HDL, high-density lipoprotein.
Mean values were significantly different from those of animals on normal diet: * $P < 0·05$, ** $P < 0·01$.
† For details of dietary treatments, see pp. 288 and 289.
‡ For details of procedures, see p. 289.

system. These zones stained positive for carbohydrate with naphthoresorcinol, and after elution were found to absorb at 259–262 nm. Further purification of the inhibitory material was attempted using a third batch of brewer's yeast which was fractionated as shown in Fig. 1.

As results with S_{10} preparations were rather variable from one preparation to another, the source of enzymes for the assay system was changed to microsomes plus ammonium sulphate fraction which could be stored at −80° without loss of activity. Table 4 shows that fraction N1 from the low-Cr yeast was more inhibitory to the incorporation of C from [1-^{14}C]acetyl-CoA than to the incorporation of mevalonate-C into non-saponifiable lipid by this microsomal preparation. Thin-layer chromatography of the non-saponifiable fractions showed that the ^{14}C was distributed between cholesterol, methyl sterols, squalene 2,3-oxide and squalene. When ^{14}C incorporation from acetyl-CoA or mevalonate was decreased by fraction N1, the percentage distribution of ^{14}C between these components was not altered. Fractions NB, NC and ND from the third batch of yeast (Fig. 1) were inhibitory to the incorporation of C from acetate or acetyl-CoA, but had a smaller effect on mevalonate incorporation (Table 4). None of the cationic fractions C1, C2 or C3 (Fig. 1) was inhibitory.

Examination of fractions NA, NB, NC and ND by thin-layer chromatography gave complex chromatograms but the eight zones found in N1 were common to all, although the proportions of the zones were not the same. Recourse was made to high-performance liquid chromatography to purify ND (Fig. 2) and seven fractions ND1–ND7 were obtained. The main inhibitory activity was found in ND2 and ND3. These fractions were examined by mass spectrometry and by proton and ^{13}C NMR. ND2 was found to be nicotinamide β-ribofuranoside. The u.v. spectrum of ND2 showed an absorption peak at 266 nm, consistent with this structure, and the absorption was shifted to 326 nm by 1 M-potassium

Table 4. *Effects of yeast fractions* on incorporation of substrate-carbon into non-saponifiable lipid by microsomes plus ammonium sulphate fraction†*

(Values are given as means with their standard errors)

Substrate...		[1-^{14}C]acetate		[1-^{14}C]acetyl-CoA		[2-^{14}C]mevalonate			
Enzyme‡		N		C		35 μM N		450 μM C	
	n	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Basal incorporation rate (μmol/h per g total protein)									
	6	0·46	0·03	—	—	2·4	0·1	—	—
	8	—	—	0·73	0·08	—	—	6·1	0·2
Percentage inhibition by yeast fractions or treated nicotinamide nucleotide (mg/ml):									
N1: 2·0	6	—	—	56	3	—	—	20	2
1·0	4	—	—	49	1	—	—	—	—
NA 2·0	6	0	2	—	—	0	3	—	—
NB 0·4	6	44	7	—	—	0	4	—	—
NC 0·3	6	34	5	—	—	16	12	—	—
ND: 1·5	6	—	—	100	1	11	4	—	—
0·5	4	37	10	—	—	5	3	—	—
ND1 9·0§	4	95	4	67	11	—	—	—	—
ND2: 0·2§	4	98	1	99	1	—	—	—	—
0·05	4	55	3	—	—	—	—	—	—
ND3: 1·2§	4	74	14	99	1	—	—	—	—
0·3	4	44	4	—	—	—	—	—	—
ND4 0·9§	4	8	2	48	3	—	—	—	—
ND5 0·2§	4	7	3	63	1	—	—	—	—
ND6 6·8§	4	12	4	—	—	—	—	—	—
ND7 0·8§	4	15	3	26	8	—	—	—	—
Treated NADH									
0·4	2	—	—	53, 57		—	—	—	—
Treated NADPH									
0·4	2	—	—	73, 74		—	—	—	—

N, animals fed on normal diet; C, animals fed on cholestyramine diet; *n*, number of replicates

* For details of fractionation procedure, see Fig. 1 and p. 286.

† For details of fractionation procedure, see pp. 289 and 290.

‡ For details of dietary treatments and extraction procedures, see pp. 288–290.

§ Amounts are proportional to the yield from high-performance liquid chromatography.

cyanide, showing the presence of the positively charged aromatic nitrogen atom. Fraction ND3, absorption maximum at 277 nm, showed only a small shift with KCN. This fraction was much less active on a weight basis than fraction ND2, and may have been contaminated with nicotinamide riboside due to the difficulty of matching exactly the collection of column effluent samples to the troughs of absorption at 254 nm. Treatment of NADH and NADPH with dilute ammonia, followed by dilute acetic acid, produced inhibitory material (Table 4) providing further support for the conclusion that one of the inhibitory materials was a nicotinamide derivative.

Attempt to find the mechanism of inhibitory action of yeast fractions

In all three assay systems, hepatocytes, S_{10} and microsomes plus ammonium sulphate fraction, the relative rates of incorporation of substrate atoms and degree of inhibition by yeast fractions were consistent with HMG-CoA reductase being the rate-limiting enzyme and site of inhibition by yeast fractions. However HMG-CoA reductase activity in

Table 5. *Hydroxymethylglutaryl-CoA (HMG-CoA) reductase* (EC *1.1.1.34*) *activity in microsomes isolated from livers of animals on various diets*, in the presence or absence of yeast fraction N1*†

(Values are given as means with their standard errors; no. of replicates four)

Diet	Substrate		HMG-CoA reductase activity (μmol/min per g microsomal protein)			
			Without fraction N1		With fraction N1	
	HMG-CoA (μM)	NADPH (mM)	Mean	SE	Mean	SE
Cholestyramine‡	330	5	1·19	0·03	1·30	0·08
	330	0·1	1·11	0·08	1·28	0·13
	33	4	0·75	0·15	0·74	0·15
Cholestyramine§	330	3‖	1·45	0·23	1·31	0·15
Normal§	330	3‖	0·27	0·02	0·26	0·01
	33	3‖	0·15	0·01	0·18	0·01
Low-chromium	330	3‖	0·32	0·03	0·31	0·03

* For details of dietary treatments, see pp. 288 and 289.
† For details of fractionation procedures, see Fig. 1 and p. 286.
‡ Microsomes prepared by the method of Kuroda & Endo (1977).
§ Microsomes prepared from S_{10} preparation.
‖ NADP plus NADPH regenerating system.

microsomes isolated from cholestyramine-fed animals was not inhibited in the presence of fraction N1 either at saturating or at rate-limiting HMG-CoA concentrations, or at 5 mM or 100 μM-NADPH (Table 5). Using microsomes isolated from S_{10} preparations from normal animals, maximum velocity (V_{max}) was lower than in microsomes from cholestyramine-fed animals, but no inhibition was seen in the presence of fraction N1 either at V_{max} or at rate-limiting concentrations of HMG-CoA.

HMG-CoA synthase and thiolase activities of the ammonium sulphate fraction were 0·85 and 0·44 μmol/min per g protein respectively, and were not inhibited by concentrations of fraction N1 which inhibited acetate incorporation into non-saponifiable lipid.

DISCUSSION

Incorporation of ^3H from tritiated water into hepatocyte lipid represents the rate of lipid synthesis from endogenous substrates in the presence of glucose. Lakshman & Veech (1977) calculated that 1·24 μmol acetyl units were incorporated into cholesterol per μg atom tritium incorporated from water; thus the observed ^3H_2O incorporation into non-saponifiable lipid (Table 1) corresponds to incorporation of 9·0 μmol acetyl units/h per g dry weight. This is in fair agreement with the value for incorporation of added [^{14}C]acetate in the presence of unlabelled substrates, whereas the value for the C_6 precurser, mevalonolactone, is equivalent to a much greater rate of incorporation of acetyl units. Also for cell-free preparations (Tables 2 and 4) relative rates of incorporation of the C_2 precursors, acetate and acetyl-CoA, compared with the C_6 precursor, mevalonate, are consistent with the rate-limiting step being between acetyl-CoA and mevalonate. Results of the one experiment using HMG-CoA as substrate suggest that it was between HMG-CoA and mevalonate, i.e. at the step catalysed by HMG-CoA reductase.

Yeast fraction N1 had a small inhibitory effect on incorporation by hepatocytes of tritium from tritiated water into non-saponifiable lipid, no effect on incorporation of

mevalonate or mevalonolactone, but strongly inhibited incorporation of acetate-C (Table 1). Lakshman & Veech (1977) showed that 66% of the ^3H incorporated into cholesterol from tritiated water was incorporated in steps after mevalonate, therefore these results are consistent with an effect of fraction N1 on the early part of the pathway between acetate and mevalonate. Results with cell-free preparations (Tables 2 and 4) support this conclusion, and the purer fractions from the third batch of yeast had relatively less inhibitory effect on melavonate incorporation than did fraction N1.

Nevertheless attempts to detect inhibition by fraction N1 of HMG-CoA reductase in microsomes were unsuccessful. Yeast fraction N1 was not competitive with HMG-CoA even at rate-limiting concentrations of HMG-CoA (Table 5). Neither were attempts to detect any kind of competition with NADPH successful. The lowest NADPH concentration used was 100 μM which is less than the $S_{0.5}$ of the native microsomal enzyme, 234 μM (Ness *et al.* 1986). However, lowering the concentration of NADPH to 100 μM did not decrease the specific activity of these preparations, so that the enzyme may have been in a proteolytically modified form for which the $S_{0.5}$ for NADPH is reported to be only 38 μM under the conditions used (Ness *et al.* 1986). Allosteric regulation of HMG-CoA reductase is complex (Roitelman & Schecter, 1984, 1986), and it may be that the failure to observe inhibition of the enzyme was related to the use of dithiothreitol in the enzyme assays whereas glutathione was the reducing agent for the incorporation experiments with S_{10} and microsomes (Tables 2 and 4) and also presumably in intact cells (Table 1). As the purest yeast fraction obtained, ND2, appears to be nicotinamide riboside, competition with NADPH would be a feasible mechanism of inhibition. It may be significant that concentrations of fraction ND2 higher than those shown in Table 4 inhibited mevalonate incorporation into non-saponifiable lipid, possibly at the later NADPH-dependent steps. Inhibition of fatty acid synthesis by fraction N1 may have a similar cause. A careful study of effects of yeast fractions on S_{10} preparations indicated that the yeast fractions were not affecting the degree of activation of the preparation during pre-incubation and, therefore, presumably not affecting dephosphorylation of phosphorylated HMG-CoA reductase or reversal of cold inactivation. The cytosolic acetoacetyl-CoA thiolase and HMG-CoA synthase, which are the first steps in the pathway of conversion of acetyl-CoA to sterols, were not inhibited by yeast fractions under the conditions used, nor was glucose-6-phosphate dehydrogenase. The possibility of effects on HMG-CoA lyase has not been excluded.

No evidence was found to support the contention that Cr may aid the lowering of blood cholesterol levels. In the present paper total cholesterol and HDL-cholesterol levels of serum were found to be identical in rats reared on normal- and low-Cr diets. However, these rats had been on the low Cr diet for only about 7 weeks which may not have been long enough for serum lipid abnormalities to develop (Schroeder, 1969), although abnormalities in body fat deposition were found (Table 3). Surprisingly, the basal rate of incorporation of acetate-C and mevalonate-C into non-saponifiable lipids was lower than normal in S_{10} preparations from low-Cr animals (Table 2), although the rate of incorporation of acetate into fatty acids was in the normal range. Estimates of HMG-CoA reductase and glucose-6-phosphate dehydrogenase have not explained this difference. Torula yeast provided a substantial part of the low-Cr diet and, like brewer's yeast, has been found to lower cholesterol levels in elderly patients (Offenbacher & Pi-Sunyer, 1980). The Torula-yeast diet was originally found to produce necrotic liver degeneration in rats (Schwarz, 1951) and the resulting defects were prevented by giving vitamin E. That diet was subsequently found to be low in Cr (Schwarz & Mertz, 1959), but other workers who have used a low-Cr diet based on casein and sucrose were unable to find an effect of the diet on plasma cholesterol (Donaldson *et al.* 1985). Possibly the Torula-yeast diet may cause a decrease in cholesterol synthesis in rat liver in vivo and in vitro, but in the present study

it did not alter the blood cholesterol levels during the 7-week period in which the rats were exposed to the diet.

The other approach to the question of Cr effects on blood lipids, i.e. comparison of inhibitory effects of fractions derived from low- and high-Cr yeast preparations, has not revealed any difference in potency for inhibition of liver cholesterol synthesis in vitro. Thus, although the experiments reported here have shown the presence in yeast extracts of substances which inhibit liver cholesterol synthesis in vitro, there is no indication of any involvement of Cr in this process. It is possible that Cr may affect blood cholesterol levels at some other site in the complex system for regulation of cholesterol levels (Brown & Goldstein, 1986). In previous investigations from this laboratory, no role for Cr could be found in the metabolism of glucose by adipocytes (Davies *et al.* 1985) or by hepatocytes (Holdsworth & Neville, 1990).

In the present paper it has been shown that extracts of yeast contain an analogue of nicotinamide nucleotides, specifically nicotinamide riboside, which inhibits incorporation of acetyl groups into non-saponifiable lipid in vitro. It has been known for some time that commercial supplies of nicotinamide nucleotides, usually prepared from yeast, contain impurities which inhibit alcohol and lactate dehydrogenases (Bernofsky & Gallacher, 1975; Loesche *et al.* 1980). It seems likely that these impurities could interfere with other enzymes requiring nicotinamide nucleotides such as those involved in synthesis of sterols and fatty acids. By deliberately exposing NADPH to alkali and acid we produced inhibitory material (Table 4). Such materials may have been present in the brewer's yeast used in clinical trials or may have been produced during intestinal digestion of nicotinamide nucleotides present in the yeast.

Another substance presence in yeast is pantethine (the disulphide of pantotheine) which has been reported to inhibit cholesterol and fatty acid synthesis (Ranganathan *et al.* 1982; Cighetti *et al.* 1986). The reported effects of pantethine differed from those of the factor reported in the present paper, since although it inhibited incorporation of acetate into cholesterol it increased incorporation into methyl sterols, thus placing the site of inhibition beyond squalene. In the present work spectroscopic evidence was obtained that pantethine was present in the fractions NA and NB. Thus, yeast appears to contain a number of substances which inhibit cholesterol synthesis by liver preparations in vitro and may contribute to the reported lowering of blood cholesterol by dietary yeast supplements. However, these factors are different from the substances that aid the utilization of a glucose load (Holdsworth & Neville, 1988).

This work was supported by a grant from the CSIRO Industrial and Equity Fund. The third batch of yeast was prepared by E.S.H. while a guest of Dr P. Buckley, Massey University, Palmerston North, New Zealand, and the high-performance liquid chromatography was done by Mr D. Doll of that University. Identification of nicotinamide riboside by nuclear magnetic resonance and mass spectrometry was the work of Dr I. C. Burgar and Dr N. Davies of the Central Sciences Laboratory, University of Tasmania, to whom the authors express their gratitude.

REFERENCES

Abraham, A. S., Sonnenblick, M. & Eini, M. (1982). The effect of chromium on cholesterol-induced atherosclerosis in rabbits. *Atherosclerosis* **41**, 371–379.

Allen, J. K., Hensley, W. J., Nicholls, A. V. & Whitfield, J. B. (1979). An enzymic and centrifugal method for estimating high-density lipoprotein cholesterol. *Clinical Chemistry* **25**, 325–327.

Bernofsky, C. & Gallacher, W. J. (1975). Lipid chromatography of pyridine nucleotides and associated compounds and isolation of several analogues of NADP. *Analytical Biochemistry* **67**, 611–624.

Bourn, D. M., Gibson, R. S., Martinez, O. B. & MacDonald, A. C. (1986). The effect of chromium supplementation on serum lipid levels in a selected sample of Canadian post-menopausal women. *Biological Trace Element Research* **9**, 197–205.

Brown, M. S. & Goldstein, J. L. (1986). A receptor-mediated pathway for cholesterol homeostasis. *Science* **232**, 34–47.

Chang, T. Y., Schiavoni, E. S. & McCrae, K. R. (1979). Inhibition of cholesterol biosynthesis in chinese hamster ovary by 4,4,10-trimethyl-trans-decal-3-ol. *Journal of Biological Chemistry* **254**, 11258–11263.

Cighetti, G., Del Puppo, M., Paroni, R., Galli, G. & Galli Kienle, M. (1986). Effects of pantethine on cholesterol synthesis from mevalonate in isolated rat hepatocytes. *Atherosclerosis* **60**, 67–77.

Conri, C. I., Simonoff, M., Besse, P., Llabador, Y., Fleury, B. & Simonoff, G. N. (1986). Baise du chrome plasmatique au cours des coronaropathies. *La Presse Médicale* **15**, 1931.

Davies, D., Holdsworth, E. S. & Sherriff, J. L. (1985). The isolation of glucose tolerance factors from brewer's yeast and their relationship to chromium. *Biochemical Medicine* **33**, 297–311.

Donaldson, D. L., Lee, D. M., Smith, C. C. & Rennert, O. M. (1985). Glucose tolerance and plasma lipid distributions in rats fed a high-sucrose, high-cholesterol, low-chromium diet. *Metabolism* **34**, 1086–1093.

Elwood, J. C., Nash, D. T. & Streeton, D. H. P. (1982). Effect of high-chromium brewer's yeast on human serum lipids. *Journal of the American College of Nutrition* **1**, 263–274.

Goodwin, C. D. & Margolis, S. (1978). Cyclic AMP-sensitive activation of hepatic sterol synthesis and 3-hydroxy-3-methylglutaryl coenzyme A reductase. *Journal of Lipid Research* **19**, 747–756.

Grant, A. P. & McMullen, J. K. (1982). The effect of brewer's yeast containing glucose tolerance factor on the response to treatment in type 2 diabetics. A short controlled study. *Ulster Medical Journal* **51**, 110–114.

Holdsworth, E. S. & Neville, E. (1988). Extracts of brewer's yeast contain GABA which enhances activation of glycogen synthetase by insulin in isolated rat hepatocytes. *Biochemistry International* **17**, 1107–1116.

Holdsworth, E. S. & Neville, E. (1990). Effects of extracts of high- and low-chromium brewer's yeast on metabolism of glucose by hepatocytes from rats fed on high- or low-chromium diet. *British Journal of Nutrition* **63**, 623–630.

Holland, R., Witters, L. A. & Hardie, G. (1984). Glucagon inhibits fatty acid synthesis in isolated hepatocytes via phosphorylation of acetyl CoA carboxylase by cyclic-AMP-dependent protein kinase. *European Journal of Biochemistry* **140**, 325–333.

Hunt, A. E., Alan, K. G. D. & Smith, B. A. (1985). Effect of chromium supplementation on hair chromium concentration and diabetic status. *Nutrition Research* **5**, 131–140.

Ingebritson, T. S. & Gibson, D. M. (1981). Assay of enzymes that modulate S-3-hydroxy-3-methylglutaryl-CoA reductase by reversible phosphorylation. *Methods in Enzymology* **71**, 486–497.

Kuroda, M. & Endo, A. (1977). Inhibition of *in vitro* cholesterol synthesis by fatty acids. *Biochimica et Biophysica Acta* **486**, 70–81.

Lakshman, M. R. & Veech, R. L. (1977). Measurement of rate of rat liver sterol synthesis *in vivo* using tritiated water. *Journal of Biological Chemistry* **252**, 4667–4673.

Layne, E. (1957). Spectrophotometric and turbidimetric methods for measuring proteins. *Methods in Enzymology* **3**, 447–454.

Li, Y.-C. & Stoecker, B. J. (1986). Chromium and yoghurt effects on hepatic lipid and plasma glucose and insulin of obese mice. *Biological Trace Element Research* **9**, 233–242.

Loesche, W., Wenz, I., Till, U., Petermann, H. & Horn, A. (1980). Purification of commercial NADH. *Methods in Enzymology* **66**, 11–22.

Lohr, G. H. & Waller, H. D. (1974). Methods for determination of enzymic activity: glucose-6-phosphate dehydrogenase. In *Methods of Enzymatic Analysis*, vol. **2**, pp. 636–643. [H. U. Bergmeyer, editor]. New York: Academic Press.

Miller, N. E., Forde, O. H., Thelle, D. S. & Mjos, O. D. (1977). The Tromso heart study. High density lipoprotein and coronary heart disease: a prospective case-control study. *Lancet* **i**, 965–968.

Ness, G. C., Sample, C. E., Smith, M., Pendleton, L. C. & Eicher, D. C. (1986). Characteristics of rat liver microsomal 3-hydroxy-3-methylglutaryl coenzyme A reductase. *Biochemical Journal* **233**, 167–172.

Newman, H. A. I., Leighton, R. F., Lanese, R. R. & Freedland, N. A. (1978). Serum chromium and angiographically determined coronary artery disease. *Clinical Chemistry* **24**, 541–544.

Offenbacher, E. G. & Pi-Sunyer, F. X. (1980). Beneficial effect of chromium-rich yeast on glucose tolerance and blood lipids in elderly subjects. *Diabetes* **29**, 919–925.

Offenbacher, E. G., Rinko, C. J. & Pi-Sunyer, F. X. (1985). The effects of inorganic chromium and brewer's yeast on glucose tolerance, plasma lipids and plasma chromium in elderly subjects. *American Journal of Clinical Nutrition* **42**, 454–461.

Potter, J. F., Levin, P., Anderson, R. A., Freiberg, J. M., Andres, R. & Elahi, D. (1985). Glucose metabolism in glucose intolerant older people during chromium supplementation. *Metabolism* **34**, 199–204.

Preston, A. M., Dowdy, R. P., Preston, M. A. & Freeman, J. N. (1976). Effect of dietary chromium on glucose tolerance and serum cholesterol in guinea pigs. *Journal of Nutrition* **106**, 1391–1397.

Rabinowitz, M. B., Gonick, H. C., Levin, S. R. & Davidson, M. B. (1983). Effects of chromium and yeast supplements on carbohydrate and lipid metabolism in diabetic men. *Diabetes Care* **6**, 319–327.

Ranganathan, S., Jackson, R. L. & Harmony, J. A. K. (1982). Effect of pantethine on the biosynthesis of cholesterol in human skin fibroblasts. *Atherosclerosis* **44**, 261–273.

Riales, R. & Albrink, M. J. (1981). Effect of chromium chloride supplementation on glucose tolerance and serum lipids including high-density lipoprotein of adult men. *American Journal of Clinical Nutrition* **34**, 2670–2678.

Roitelman, J. & Schecter, I. (1984). Regulation of rat liver 3-hydroxy-3-methylglutaryl coenzyme A reductase. *Journal of Biological Chemistry* **259**, 870–877.
Roitelman, J. & Schecter, I. (1986). Altered kinetic properties of rat liver 3-hydroxy-3-methylglutaryl coenzyme A reductase following dietary manipulations. *Journal of Biological Chemistry* **261**, 5061–5066.
Schroeder, H. A. (1969). Serum cholesterol and glucose levels in rats fed refined and less refined sugars and chromium. *Journal of Nutrition* **97**, 237–242.
Schwarz, K. (1951). Production of dietary necrotic liver degeneration using American Torula yeast. *Proceedings of the Society for Experimental Biology and Medicine* **77**, 818–823.
Schwarz, K. & Mertz, W. (1959). Chromium III and the glucose tolerance factor. *Archives of Biochemistry and Biophysics* **85**, 292–295.
Simonoff, M., Llabador, Y., Hamon, C., Peers, A. M. & Simonoff, G. N. (1984). Low plasma chromium in patients with coronary artery and heart disease. *Biological Trace Element Research* **6**, 431–439.
Smith, I. (1969). *Chromatographic and Electrophoretic Techniques*. vol. 1, 3rd ed., p. 104. Bath: Pitman.
Stoecker, B. J. & Oladut, W. K. (1985). Effects of chromium and ascorbate deficiencies on glucose tolerance and serum cholesterol of guinea pigs. *Nutrition Reports International* **32**, 399–405.
Uusitupa, M. I. J., Kumpulainen, J. T., Voutilainen, E., Hersio, K., Sarlund, H., Pyorala, K. P., Kiovistoinen, P. E. & Lehto, J. T. (1983). Effect of inorganic chromium supplementation on glucose tolerance, insulin response, and serum lipids in non-insulin-dependent diabetes. *American Journal of Clinical Nutrition* **38**, 404–410.
Vinson, J. A. & Bose, P. (1984). The effect of a high-chromium yeast on the blood glucose control and blood lipids of normal and diabetic human subjects. *Nutrition Reports International* **30**, 911–918.
Wallach, S. (1985). Clinical and biochemical aspects of chromium deficiency. *Journal of the American College of Nutrition* **4**, 107–120.

Printed in Great Britain