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Stimulation of Liver Cholesterol Synthesis by Actinomycin D

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1. An eightfold increase in the incorporation of [2-^{14}C]acetate into liver cholesterol *in vivo* was observed 24hr. after starved rats had been given actinomycin D (0·5mg./kg. of body wt.). Liver cholesterol radioactivity declined faster in the treated animals, suggesting a greater rate of cholesterol turnover. 2. Liver slices from treated animals showed a tenfold increase in the incorporation of [2-^{14}C]acetate into cholesterol; conversion into CO_2 and into fatty acids was less markedly increased, and conversion into ketone bodies was not significantly affected. 3. The patterns of conversion into liver cholesterol *in vivo* of the lactone and the sodium salt of mevalonic acid differed markedly. The former was converted at a faster rate and to a greater extent than the latter. Treatment with actinomycin D increased the conversion of both forms of mevalonic acid into liver cholesterol, but only to a small extent. 4. Stimulation of the incorporation of acetate into cholesterol occurred at 4hr. after the administration of actinomycin D but not at 2hr. The response was abolished by the simultaneous administration of DL-ethionine or puromycin. 5. Pre-feeding with a cholesterol-rich diet greatly diminished the stimulation of conversion of acetate into cholesterol caused by actinomycin D, though it did not completely suppress it. Adrenalectomized animals responded to the drug, but much less markedly. 6. It is concluded that actinomycin D stimulates the synthesis of cholesterol in the liver at a stage in the pathway before mevalonic acid, by a mechanism that probably requires protein synthesis. A likely site would be the β-hydroxy-β-methylglutaryl-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis. Some possible mechanisms by which the drug may lead to increased activity of this enzyme are considered.

In an earlier study it was shown that the administration of actinomycin D to the rat caused a rise in serum cholesterol concentration (De Matteis, 1966*a*). Such a rise could be produced by an increase in the rate of synthesis of cholesterol, by a decrease in its rate of breakdown, or by a redistribution of cholesterol from other body compartments in favour of the plasma. Since the liver plays an important role in both synthesis (Hotta & Chaikoff, 1955) and degradation (Friedman, Byers & Gunning, 1953) of serum cholesterol, the effect of the administration of actinomycin D on liver cholesterol metabolism was studied. A preliminary report of some of the findings has appeared (De Matteis, 1966*b*).

MATERIALS AND METHODS

Treatment of animals

Male albino rats of the Porton strain were used. The rats weighed 120–150g., except for the animals for the labelling experiments *in vivo*, which weighed 45–60g. Adrenalectomized animals were used 6 days after adrenalectomy; they were given 0·9% NaCl to drink instead of tap water. These animals were checked for accessory cortical tissue at death and none was found. Cube diet M.R.C. 41B (Bruce & Parkes, 1956) was available to the rats until the start of the experiments, when it was withdrawn to avoid variations in food intake due to the treatment. During the starvation period the rats were kept at 18–22° with water freely available, in individual cages designed to prevent coprophagy. Actinomycin D (0·1mg./ml. of 0·9% NaCl) was injected in the loose subcutaneous tissue of the neck. DL-Ethionine (Calbiochem, Los Angeles, Calif., U.S.A.) was given by stomach tube in aqueous suspension (50mg./ml.) and puromycin hydrochloride (Nutritional Biochemical Corp., Cleveland, Ohio, U.S.A.) was administered by intraperitoneal injection suspended in buffered saline (0·04M-Na_2HPO_4–NaH_2PO_4, pH 7·4, in 0·9% NaCl) at a concentration of 17mg./ml. Control animals were given the solvent alone by injection or by feeding, as appropriate. The rats were killed by guillotine.

In the labelling experiments *in vivo*, [2-^{14}C]acetate (6μc/100g. body wt.), DL-[2-^{14}C]mevalonic acid (0·5μc/100g.), either as the lactone or as the sodium salt, or [6-^{14}C]orotate (2μc/100g.) was injected intraperitoneally.

The labelled compounds were obtained from The Radiochemical Centre, Amersham, Bucks., and had the following specific radioactivities: [2-^{14}C]acetate, 33–38mc/m-mole; [2-^{14}C]mevalonic acid, 5·03mc/m-mole; [6-^{14}C]orotate, 44·5mc/m-mole. The benzene solution of the mevalonic acid lactone was evaporated to dryness under a gentle stream of N_2 and the lactone dissolved in 0·9% NaCl: this saline solution was used for injection within 1hr. of preparation. Sodium mevalonate was prepared by hydrolysing the lactone by the addition of dil. NaOH until the pH remained slightly alkaline (pH 7–8).

Incubation of liver slices

For the experiments *in vitro*, slices approx. 0·4mm. thick were cut by hand from the left lobe of the liver, gently blotted and weighed. Liver slices (approx. 500mg.) were incubated for 1hr. with 12·3μmoles (1μc) of [2-^{14}C]acetate or with 2·5μmoles (0·5μc) of sodium DL-[2-^{14}C]mevalonate in 3·5ml. of Krebs–Ringer phosphate solution, pH 7·4 (Umbreit, Burris & Stauffer, 1959), containing $CaCl_2$ (1·35mM). The incubations were carried out at 37·4° in a Warburg flask containing alkali in the centre well, with O_2 as the gas phase and with shaking (95–100 double oscillations/min.). Under these conditions the incorporation of both labelled acetate and labelled mevalonate into cholesterol and the conversion of [2-^{14}C]acetate into $^{14}CO_2$ proceeded at a linear rate for at least 2hr., and the amount of label from [2-^{14}C]acetate recovered in either $^{14}CO_2$ or liver cholesterol increased regularly with the weight of liver slices over the whole range examined (72–504mg.).

At the end of the incubation the contents of the flasks were acidified by the injection into each flask of 0·45ml. of 0·66N-H_2SO_4 through a rubber stopper on the side arm. When conversion of [2-^{14}C]acetate into acetone and acetoacetate was measured, after the addition of H_2SO_4 the flask was transferred to an ice bath and 10mg. of carrier acetone was added [this is about 500 times more than the amount of acetoacetate present in 500mg. of liver of rats starved for 24hr. (Berry, Williamson & Wilson, 1965)]. When conversion of [2-^{14}C]acetate into $^{14}CO_2$ was measured, the addition of H_2SO_4 was followed by a further incubation of 30min. (this resulted in a quantitative recovery of ^{14}C from standard [^{14}C]carbonate in the alkali of the centre well of the flask).

Techniques for experiments with labelled compounds

Isolation of labelled cholesterol and fatty acids. The livers from animals injected with labelled acetate or mevalonate and the liver slices from experiments *in vitro* were digested as described by Entenman (1957), except that the quantity of KOH and the volume of the digesting mixture were increased to approx. 1g. and 10ml./g. of liver tissue respectively. Cholesterol was separated from fatty acids and both were extracted into light petroleum (b.p. 60–80°) as described by Entenman (1957).

The cholesterol fraction was evaporated to dryness in a rotary evaporator. The residue was dissolved in 3·5ml. of ethanol, and the ethanolic solution was filtered through a defatted cotton-wool plug into 3ml. of acetone. Then 3ml. of 0·5% digitonin solution in 50% (v/v) ethanol was added and, 1hr. later, the digitonide of cholesterol was collected by centrifugation, washed with 8ml. of ethanol–acetone (1:1, v/v), dried under a stream of air and dissolved in 1ml. of acetic acid. A sample (0·5ml.) of the solution was transferred to a scintillation vial for counting and a sample (0·2ml.) taken for colorimetric determination of cholesterol. Further purification of the radioactive cholesterol from both control and treated animals by splitting the digitonide (Schoenheimer & Dam, 1933), crystallizing the dibromo derivative, 5α,6β-dibromocholestan-3β-ol, regenerating cholesterol (Kabara & McLaughlin, 1961) and isolating it again as the digitonide did not significantly alter its specific radioactivity.

The light-petroleum solution of labelled fatty acids was evaporated to dryness in a tared scintillation vial, and the fatty acids were weighed and dissolved in 0·5ml. of toluene for counting.

Isolation of labelled acetone. After the addition of carrier acetone the liver slices and incubation medium were homogenized in the cold. Then 10% (w/v) sodium tungstate (0·45ml.) was added and, after centrifugation, the supernatant was transferred to a flask containing 30ml. of water and 3ml. of conc. H_2SO_4. The flask was connected to a distillation apparatus and heated until about 15ml. of distillate had been collected. Under these conditions the acetoacetate present was decarboxylated to yield acetone and the acetone was quantitatively recovered in the distillate; the label present in β-hydroxybutyrate was not determined by this procedure. A sample of the distillate was then treated with Denigès's reagent for 90min. in a boiling-water bath under a reflux condenser (Weichselbaum & Somogyi, 1941). The precipitated acetone–mercury complex was washed with three 5ml. volumes of water by centrifugation and dissolved in 5ml. of 5N-HCl. Then 5ml. of (0·5%) DNP-hydrazine in 2N-HCl was added and, after 5min., the DNP-hydrazone was extracted from the water phase with small amounts of CCl_4. The CCl_4 extracts were pooled, and washed twice with the same volume of water, once for 1min. with a small volume of 0·5N-NaOH, and again with water, and finally dried by filtration through three layers of filter paper wetted with CCl_4. A suitable dilution of the DNP-hydrazone solution was read at 349mμ and compared with standard dilutions of acetone DNP-hydrazone, and a volume of CCl_4 solution containing no more than 70μg. of DNP-hydrazone was evaporated to dryness in a scintillation vial: under these conditions quenching was negligible and counting efficiency exceeded 60%. No change in specific radioactivity was observed after crystallization of the acetone DNP-hydrazone from aqueous ethanol.

Isolation of labelled carbon dioxide. When conversion of [2-^{14}C]acetate into $^{14}CO_2$ by liver slices was measured, the contents of the central well of the flasks were transferred to a 50ml. centrifuge tube. The centre well was rinsed first with 1ml. of 0·1N-NaOH–0·2M-Na_2CO_3 and then with 5ml. of water, and the rinsings were added to the tube, followed by 10ml. of 10% (w/v) $BaCl_2$. The mixture was heated for 10min. in a boiling-water bath and allowed to cool, and the pH was adjusted with 0·1N-HCl to the phenolphthalein end point. The $BaCO_3$ was collected by centrifugation, washed with 40ml. of water and resedimented; the supernatant was discarded by draining off as much water as possible.

Preparation of RNA for counting. When the incorporation of [6-^{14}C]orotate into liver RNA *in vivo* was studied, the animals were killed 30min. after injection of the label. A sample of the liver homogenate containing labelled RNA

was repeatedly extracted with cold 7% (w/v) trichloroacetic acid and with ethanol–ether. The RNA was hydrolysed at 37° for 18hr. in 0·3N-NaOH and, at the end of this period, the hydrolysate was acidified and trichloroacetic acid was added to a concentration of 5% (w/v) to precipitate DNA. A sample (0·5ml.) of the supernatant was transferred to a scintillation vial for counting and another sample was used for the determination of RNA. In one experiment the RNA was also isolated by a phenol method (Parish & Kirby, 1966), with essentially similar results.

Counting procedure. Portions (10ml.) of a scintillator fluid [a 1:3:3 (by vol.) toluene–dioxan–ethyl Cellosolve mixture containing 1% of 2,5-diphenyloxazole, 0·05% of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene and 8% of naphthalene] were added to the vials containing labelled cholesterol, fatty acids, acetone DNP-hydrazone or RNA hydrolysate for counting. The labelled $BaCO_3$ was transferred to a scintillation vial with three 5ml. portions of the scintillator fluid containing 4% of Cab-O-Sil (Packard Instrument Co., La Grange, Ill., U.S.A.).

All samples were counted in a Packard Tri-Carb liquid-scintillation spectrometer and corrected for quenching by the use of internal standards.

Analytical methods

Total liver lipids were determined gravimetrically by the method of Folch, Ascoli, Lees, Meath & Le Baron (1951). Samples of the washed total lipid extract were used to determine total cholesterol (Abell, Levy, Brodie & Kendall, 1952). The method of Abell *et al.* (1952) was also followed for the determination of cholesterol digitonide from the radioactive samples. Ash-free cholesterol precipitated from ethanol [Sigma Chemical (London) Co., London, S.W. 6] was used as standard. RNA was determined by the orcinol procedure (Mejbaum, 1939); yeast RNA, purified by the method of Crestfield, Smith & Allen (1955), was used as the standard. The water content of the liver was determined by drying a small sample overnight at 105°.

Calculation of results

Results are generally expressed as the arithmetic means ±S.E.M. and the means compared by Student's t test as modified by Fisher (1934) for small samples. When the experimental values were widely scattered and appeared to deviate considerably from a normal distribution, results are expressed as the geometric means (with S.E. range) and the logarithms of the values used for Student's t test (Heath, 1967).

RESULTS

Labelling studies in vivo

Effect of actinomycin D on the incorporation of labelled acetate into liver cholesterol and fatty acids. The incorporation of labelled acetate into liver cholesterol and fatty acids and the rate of decline of radioactivity of liver cholesterol were studied *in vivo* in actinomycin D-treated animals and in their controls (Fig. 1 and Table 1). Incorporation of acetate into liver cholesterol after intraperitoneal injection was very rapid, and the highest specific radioactivity was at 15min. in both control and treated animals; the specific radioactivity of the liver cholesterol at this time was over eight times greater in the treated animals (Table 1). Thereafter the specific radioactivity declined in both groups.

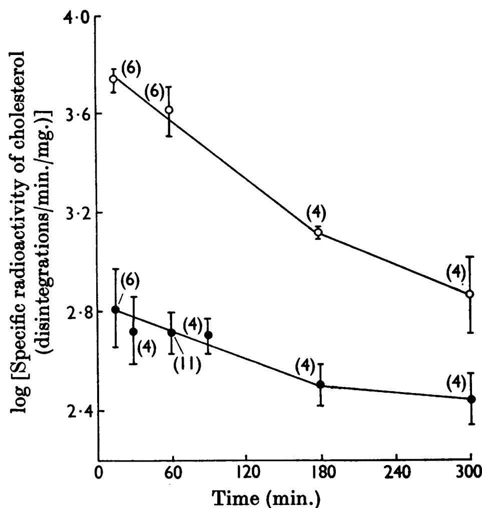

Fig. 1. Specific radioactivity of liver cholesterol at different times after intraperitoneal injection of [2-^{14}C]acetate to control (●) and actinomycin D-treated (○) rats. Each point represents the mean ± S.E.M. of the logarithms of the values observed. The numbers of observations are given in parentheses. The animals were treated as described in Table 1.

Table 1. *Effect of treatment with actinomycin D on the incorporation of* [2-^{14}C]*acetate into liver cholesterol and fatty acids* in vivo *by the rat*

The use of geometric means and S.E. range is discussed in the Materials and Methods section. Actinomycin D (0·5mg/kg.) was given to six rats by subcutaneous injection in two equal doses in the first 4hr.; 24hr. after the first dose the rats were injected intraperitoneally with [2-^{14}C]-acetate (6μc/100g. body wt.) and killed 15min. later. All rats were starved for 24hr. before injection of the labelled compound.

Treatment	Sp. radioactivity of liver cholesterol (disintegrations/min./mg.) Geometric mean	S.E. range	Sp. radioactivity of liver fatty acids (disintegrations/min./mg.) Mean ± S.E.M.
Control	647	448–935	98 ± 16
Actinomycin D	5420	4820–6095***	154 ± 15*

*$P<0·05$; ***$P<0·001$, when compared with the corresponding control.

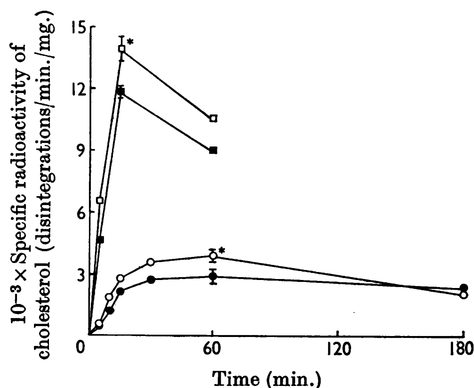

Fig. 2. Specific radioactivity of liver cholesterol at different times after intraperitoneal injection of DL-[2-^{14}C]mevalonic acid lactone (■, controls; □, actinomycin D-treated animals) or of sodium DL-[2-^{14}C]mevalonate (●, controls; ○, actinomycin D-treated animals). The animals were treated as described in Table 1. Each point represents the mean of at least four observations for sodium mevalonate and of at least three for mevalonic acid lactone. At the points of highest specific radioactivity the S.E. of the means is also given. $*P<0{\cdot}05$, when compared with the corresponding control.

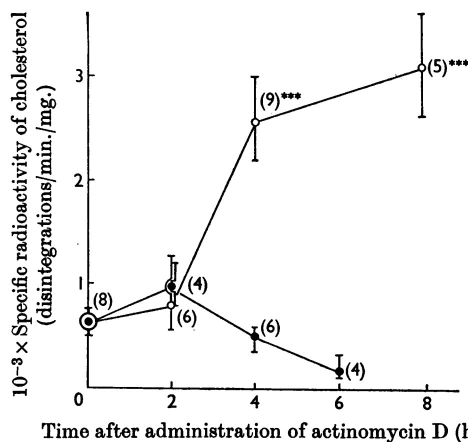

Fig. 3. Incorporation of [2-^{14}C]acetate into cholesterol *in vivo* at various time-intervals after a single actinomycin D dose of either 0·6mg./kg. body wt. (○) or 1·2mg./kg. body wt. (●). Each point represents the geometric mean with S.E. range of the values observed. The numbers of observations are given in parentheses. $***P<0{\cdot}001$, when compared with the values observed in animals not given any drug (zero time in the Figure). All animals were killed 15min. after injection of the labelled compound and starved for 24hr. before being killed.

The rate of decline was appreciably greater in the actinomycin D-treated animals; the gradients between 15 and 180min. and errors on them were estimated by standard methods (least-mean-square fit) and were significantly different ($P\leqslant 0{\cdot}001$): this suggested a greater rate of turnover of liver cholesterol in the treated animals.

The incorporation of labelled acetate into liver fatty acids was also increased at 15min. in the treated animals, but less markedly (Table 1).

Effect of actinomycin D on the incorporation of labelled mevalonic acid into cholesterol. Elwood & Van Bruggen (1961) studied the conversion of DL-[2-^{14}C]mevalonic acid into liver cholesterol in the rat *in vivo* and found that maximum specific radioactivity was attained within 30min. of the injection of the label. It is not clear whether free mevalonic acid or its lactone was injected in their study, though the results of the present work suggest that a large proportion may have been the lactone.

A very marked difference exists between the lactone and the sodium salt of mevalonic acid in the pattern of their conversion into liver cholesterol *in vivo* (Fig. 2). With sodium mevalonate, conversion into liver cholesterol was slow, maximum specific radioactivity being reached at about 1hr. after injection: at this time approx. 3% of the injected label was recovered in liver cholesterol. With the lactone of mevalonic acid, conversion into liver cholesterol occurred faster and to a greater extent: thus at 15min. after injection about 12% of the total dose was in liver cholesterol. This difference between the lactone and the sodium salt of mevalonic acid suggests that the two forms may differ in the rate at which they reach the site within

Table 2. *Stimulation of the incorporation of* [2-^{14}C]-*acetate into liver cholesterol* in vivo *caused by actinomycin D: dose–response study*

A single dose of progressively increasing quantities of actinomycin D was given subcutaneously to rats that had been starved for 20hr. After 4hr. they were given an intraperitoneal injection of [2-^{14}C]acetate (6μc/100g. body wt.) and killed 15min. later.

Dose of actinomycin D (mg./kg. body wt.)	Sp. radioactivity of liver cholesterol (disintegrations/min./mg.) No. of observations	Geometric mean	S.E. range
Nil (Control)	8	621	503–766
0·1	3	459	350–601
0·3	5	1702	1021–2838
0·6	9	2559***	2188–2992
1·2	6	455	347–595

$***P<0{\cdot}001$, when compared with the control.

the liver where they are further converted: this might depend on a difference in their lipid/water partition coefficient, a property known to govern the diffusion of several substances across biological membranes (Boyce & Milborrow, 1965): the lactone would be expected to diffuse more readily than the sodium salt of the free acid.

In contrast with the results obtained with labelled acetate, the incorporation of mevalonate was only slightly, though significantly, increased above control values after treatment with actinomycin D, and the increase caused by the drug was of the same order of magnitude whether sodium mevalonate or mevalonic acid lactone was used (Fig. 2).

Time-course of the stimulation of the conversion of labelled acetate into cholesterol by actinomycin D and dose–response study. Animals were given a single subcutaneous dose of actinomycin D (0·6mg./kg.); after various intervals they were injected intraperitoneally with [2-^{14}C]acetate and killed 15min. later. The time schedule of this and of all the following experiments *in vivo* was adjusted so that all animals (including control animals given injections of 0·9% sodium chloride) had been starved for 24hr. by the time of the injection of the labelled compound. The effect of actinomycin D in stimulating the incorporation of acetate into cholesterol was fairly rapid but not immediate (Fig. 3). The incorporation of acetate into cholesterol was significantly stimulated at 4hr., but no effect was seen at 2hr.

When a single dose of progressively increasing quantities of the drug was given 4hr. before injection of the labelled compound, it was found (Table 2) that the effect was related to the amount administered: the optimum dosage required for the reaction was about 0·6mg./kg.; 1·2mg./kg. did not increase the incorporation of acetate into cholesterol (see also Fig. 3). Animals given 1·2mg. of the antibiotic/kg. were the only ones to show signs of toxicity in the form of dilated stomach and intestine with excessive fluid content.

Effect of inhibitors of RNA and protein synthesis. Actinomycin D is known to inhibit the DNA-directed synthesis of RNA, and so the incorporation of [6-^{14}C]orotate into rapidly labelled liver RNA was measured under the conditions of the experiments described in Table 2. A dose of the drug (0·6mg./kg.) that stimulated the conversion of acetate into cholesterol inhibited the incorporation of orotate into RNA by 34%.

After the injection of 0·6mg. of actinomycin D/kg., the stimulation of acetate incorporation into cholesterol was abolished when either DL-ethionine or puromycin was administered with actinomycin D (Table 3). This was unlikely to have resulted either from an effect of the former two drugs on absorption of the label from the peritoneal cavity, or from a direct inhibition of the incorporation of labelled acetate into liver cholesterol, since such treatment did not significantly inhibit the incorporation of labelled acetate into liver cholesterol by control animals, nor did puromycin decrease the incorporation of the label into liver fatty acids in either group of rats.

Experiments in vitro

Effect of actinomycin D treatment on the metabolism of acetate and mevalonate in vitro. The conversion of [2-^{14}C]acetate into carbon dioxide, cholesterol,

Table 3. *Effect of* DL-*ethionine and of puromycin on the stimulation of conversion of* [2-^{14}C]*acetate into liver cholesterol caused by actinomycin D*

DL-Ethionine was given in two doses, 2hr. and 1hr. before actinomycin D; puromycin was given in four doses, 20min. before actinomycin D and hourly thereafter; actinomycin D was administered in a single dose of 0·6mg./kg; 4hr. after administration of actinomycin D, the rats were injected intraperitoneally with [2-^{14}C]acetate and killed 15min. later. All rats were starved for 24hr. before injection of the labelled compound.

			Sp. radioactivity of liver cholesterol			Sp. radioactivity of liver fatty acids	
Group	Inhibitor and total dose administered		No. of observations	disintegrations/min./mg. (geometric mean [S.E. range])	Actinomycin D / Control	No. of observations	disintegrations/min./mg. (mean ± S.E.M.)
1	None	Control	5	350 [174–703]	6·1	4	122 ± 6
		Actinomycin D	5	2138* [1722–2655]			
2	DL-Ethionine (1g./kg.)	Control	6	444 [309–637]	0·61		
		Actinomycin D	5	271 [197–374]			
3	Puromycin (0·2g./kg.)	Control	4	229 [185–285]	1·79	4	123 ± 12
		Actinomycin D	4	410 [289–582]		4	149 ± 16

* $P < 0.05$, when compared with the controls of the same group.

long-chain fatty acids, and acetone and acetoacetate, and the incorporation of sodium DL-[2-^{14}C]-mevalonate into cholesterol, were measured *in vitro*.

Liver slices from rats that had been treated with actinomycin D incorporated [2-^{14}C]acetate into cholesterol over ten times more actively than did liver slices from control animals (Table 4). Similar degrees of stimulation were obtained when the amount of the substrate acetate was increased to 36 and 123μmoles (from the 12·3μmoles usually used) or when glucose (36μmoles) was included in the incubation medium. Actinomycin D added *in vitro* to slices from starved control animals in concentrations ranging from 0·03 to 14μg./ml. did not stimulate the incorporation of [2-^{14}C]acetate into cholesterol: at the highest concentrations it inhibited oxygen uptake.

The incorporation of [2-^{14}C]acetate into fatty acids and ketone bodies was not significantly affected after actinomycin D treatment; however, conversion into carbon dioxide was slightly but significantly increased above the control value (Table 4). Incorporation of mevalonate into cholesterol by liver slices was only slightly increased after treatment with actinomycin D; the increase was not statistically significant (Table 4).

In the work described so far, all the animals were starved for 24hr. Starvation is known to cause a marked decrease in the rate of synthesis of liver cholesterol (Tomkins & Chaikoff, 1952) and fatty acids (Medes, Thomas & Weinhouse, 1952). For example, liver slices from rats that had been starved for 24hr. showed a ten- and 40-fold decrease in the incorporation of [2-^{14}C]acetate into cholesterol and fatty acids respectively compared with liver slices from fed animals (Table 5). Actinomycin D-treated animals passed less faeces than control animals, and their stomachs after 24hr. contained traces of food at the time of killing. The possibility was therefore considered that actinomycin D might have prevented the decrease in lipid synthesis associated with starvation by delaying the absorption of foodstuffs, and that this might have given rise to an apparent stimulation of the conversion of [2-^{14}C]acetate into cholesterol. However, when rats were starved for 24hr. before actinomycin D was administered, and killed after a further 24hr. of starvation, their liver slices incorporated [2-^{14}C]acetate into cholesterol at a significantly greater rate than did liver slices from control animals that had been starved for only 24hr. (Table 5). A significantly increased incorporation of acetate into fatty acids was also observed in the liver slices from animals treated with actinomycin D and starved for 48hr.

The livers from animals treated with actinomycin D did not differ from those of controls in size, dry weight or cholesterol and total lipid content.

Table 4. *Incorporation of* [2-^{14}C]*acetate and of* DL-[2-^{14}C]*mevalonate into liver cholesterol and the conversion of* [2-^{14}C]*acetate into fatty acids, into carbon dioxide and into acetone and acetoacetate by liver slices from animals treated with actinomycin D and by liver slices from control animals*

Actinomycin D (0·5mg./kg.) was given by subcutaneous injection in two equal doses 24 and 20hr. before the animals were killed. Animals were starved for 24hr.

Labelled compound	Treatment	Radioactivity recovered (disintegrations/min.): In cholesterol, No. of observations	In cholesterol, Geometric mean [S.E. range]	In fatty acids, No. of observations	In fatty acids, Mean ± S.E.M.	In CO_2, No. of observations	In CO_2, Mean ± S.E.M.	Sp. radioactivity (disintegrations/min./mg.) of acetone DNP-hydrazone, No. of observations	Sp. radioactivity, Mean ± S.E.M.
[2-^{14}C]Acetate	Control	6	1849 [1225–2793]	6	511±74	8	136725±6394	6	18187±949
	Actinomycin D	5	27670*** [21380–35810]	5	784±116	7	179671**±8114	6	14898±1248
DL-[2-^{14}C]Mevalonate	Control	5	91410 [77980–106700]	—	—	—	—	—	—
	Actinomycin D	5	125600 [116700–135200]	—	—	—	—	—	—

** $P < 0·01$; *** $P < 0·001$, when compared with the corresponding controls.

Table 5. *Effect of the nutritional state of the rat on the incorporation of* [2-^{14}C]*acetate into cholesterol and fatty acids by liver slices and on the response to actinomycin D treatment*

Animals were treated with actinomycin D as described in Table 4.

		Radioactivity recovered (disintegrations/min.)			
		In cholesterol		In fatty acids	
Nutritional state at time of killing	Treatment	No. of observations	Geometric mean [S.E. range]	No. of observations	Mean ± S.E.M.
Fed	None	8	20700 [15900–26920]	4	22617 ± 8803
Starved for 24hr.	Control	6	1849 [1225–2793]	6	511 ± 74
	Actinomycin D	5	27670*** [21380–35810]	5	784 ± 116
Starved for 48hr.	Control	5	374 [204–685]	5	431 ± 57
	Actinomycin D	5	16750***††† [12970–21630]	4	806* ± 113

The values marked with asterisks differ significantly from the controls of the same group (* $P<0.05$; *** $P<0.001$); the value marked with daggers differs significantly from the controls that were starved for 24hr. (††† $P<0.001$].

When the animals were starved for 48hr. a slight increase in liver size, with a small decrease in liver cholesterol content/g. wet wt. of liver, was noted in the treated animals.

Effect of dietary cholesterol and of adrenalectomy on the increased conversion of acetate into cholesterol caused by actinomycin D. Cholesterol is known to exercise a feedback control on its own synthesis: increased dietary intake of cholesterol results in a marked depression of liver cholesterol synthesis, as measured by the incorporation of labelled acetate into cholesterol (Tomkins, Sheppard & Chaikoff, 1953). Experiments were carried out to test whether the stimulation of [2-^{14}C]acetate incorporation into cholesterol caused by actinomycin D could be prevented by feeding with excess of cholesterol. Experiments were also performed with adrenalectomized animals to find whether the effect of actinomycin D was mediated through an endocrine response involving the adrenal glands.

Rats were fed on powder diet M.R.C. 41B containing 2% of cholesterol for either 1, 2 or 3 days. They were then treated with actinomycin D and starved for 24hr., and the conversion of labelled acetate into cholesterol by liver slices prepared from them was studied. Prefeeding with cholesterol greatly diminished the stimulation of acetate conversion into cholesterol caused by actinomycin D, though it did not completely suppress it (Table 6*a*). Inhibition of the conversion of labelled acetate into cholesterol was most marked in the actinomycin D-treated animals. The concentration of cholesterol in the liver and the rate of incorporation of labelled acetate into cholesterol were inversely related as reported by Frantz, Schneider & Hinkelman (1954). However, a few animals in both control and treated groups that did not have an increased liver cholesterol concentration did nevertheless exhibit a very significantly decreased incorporation of acetate into cholesterol (Table 6*b*): here again the degree of inhibition caused by prefeeding with cholesterol was greater in the actinomycin D-treated animals.

Adrenalectomy markedly decreased the increased incorporation of acetate into cholesterol caused by actinomycin D, but did not completely suppress it (Table 7).

DISCUSSION

Stimulation of cholesterol synthesis by actinomycin D. The present work has shown that increased incorporation of acetate into cholesterol *in vivo* follows the administration of actinomycin D. The most likely explanation for this finding is that the rate of synthesis of cholesterol in the liver is stimulated by this drug. There are three lines of evidence that favour this interpretation. (1) After actinomycin D treatment an increase in the incorporation of acetate into cholesterol (quantitatively similar to that observed *in vivo*) was found *in vitro*, under conditions where the concentration of the labelled substrate did not appear to be limiting; no significant changes were observed in the incorporation of labelled acetate into acetone and acetoacetate and only slight increases (compared with the effects on cholesterol) were found in the conversion of the label into fatty acids and carbon dioxide. Both

Table 6. *Effect of feeding a cholesterol-rich diet on the incorporation of* [2-^{14}C]*acetate into cholesterol by liver slices and on increased incorporation caused by actinomycin D*

Rats were fed on powder diet M.R.C. 41B containing 2% of cholesterol for either 1, 2 or 3 days and then treated with actinomycin D, as described in Table 4, and starved for 24hr. Cholesterol-fed animals are grouped according to the duration of cholesterol feeding (*a*), or according to the liver cholesterol concentration found at death (*b*).

Duration of cholesterol feeding (days)	Treatment	No. of observations	Liver cholesterol (mg./100g.) (mean ± S.E.M.)	Radioactivity recovered in cholesterol (disintegrations/min.): Geometric mean [S.E. range]	Actinomycin D / Control	Inhibition caused by feeding with cholesterol (%)
(*a*)						
0	Control	6	276 ± 40	1849 [1225–2793]	14·96	—
	Actinomycin D	5	263 ± 4	27670*** [21380–35810]		—
1	Control	5	341 ± 57	268 [123–586]	3·19	85·5
	Actinomycin D	5	354 ± 49	855 [570–1282]		96·9
3	Control	5	562 ± 118	227 [153–338]	2·5	87·7
	Actinomycin D	5	508 ± 106	570 [312–1042]		97·9
(*b*) Group 1 (normal liver cholesterol content)	Control	4	258 ± 24	887 [841–1507]	2·77	52·0
	Actinomycin D	4	269 ± 8	2460 [2023–2992]		91·1
Group 2 (increased liver cholesterol content)	Control	7	567 ± 72	92 [74–114]	3·36	95·0
	Actinomycin D	8	532 ± 61	390** [241–396]		98·9

** $P < 0{\cdot}01$; *** $P < 0{\cdot}001$, when compared with the corresponding controls.

Table 7. *Effect of adrenalectomy on the incorporation of* [2-^{14}C]*acetate into cholesterol by liver slices from animals treated with actinomycin D and from their controls*

Animals were treated with actinomycin D as described in the legend of Table 4, and starved for 24hr.

Treatment	No. of observations	Radioactivity recovered in cholesterol (disintegrations/min.) (geometric mean [S.E. range])
Control	8	274·8 [202–374]
Actinomycin D	8	873* [615–1239]

*$P < 0·05$ when compared with the control value.

these observations suggest that the increase in cholesterol radioactivity found after actinomycin D treatment is more likely to result from a metabolic response to the drug involving cholesterol biosynthesis than from a decrease in the size of the acetate pool or from a generalized increase in the utilization of acetate. (2) An increased conversion of labelled mevalonic acid into liver cholesterol was also observed. (3) Evidence for an increased rate of turnover of liver cholesterol was obtained in the experiments *in vivo*: this also suggests an increased rate of synthesis of liver cholesterol.

It must be emphasized, however, that the conversion of labelled acetate into carbon dioxide and fatty acids was also increased after actinomycin D treatment, indicating that the effect on the biosynthesis of cholesterol, though much more pronounced, was not the only metabolic change caused by the drug.

Site in the pathway of cholesterol biosynthesis where stimulation takes place and effect of dietary cholesterol. The increase in acetate incorporation into cholesterol was considerably greater than the increase in mevalonate incorporation. This suggests that the major site of stimulation was at some point in the biosynthetic pathway before mevalonate. A likely site would be the microsomal enzyme, HMG-CoA* reductase (EC 1.1.1.34), the rate-limiting enzyme in cholesterol biosynthesis. This enzyme catalyses the conversion of HMG-CoA [which is also the direct precursor of acetoacetate (Lynen, Henning, Bublitz, Soerbo &Kroeplin-Rueff, 1958; Sauer & Erfle, 1966)] into mevalonate, an essentially irreversible reaction (Knappe, Ringelmann & Lynen, 1959), which is the first step on the direct pathway to cholesterol (Lynen *et al.* 1958) and which appears to be the main site of the physiological control of cholesterol synthesis.

The synthesis of cholesterol (as measured by the incorporation of labelled acetate into cholesterol) is considerably inhibited by feeding with cholesterol (Tomkins *et al.* 1953) or by starvation (Tomkins & Chaikoff, 1952), and it is increased by X-irradiation (Gould, Bell & Lilly, 1956), by intravenous injection of Triton WR-1339 (Frantz & Hinkelman, 1955) or by physical injury (De Matteis, 1968). In all these conditions and in the present work, comparison of the incorporation into cholesterol of labelled acetate with the incorporation of mevalonate (Gould & Popják, 1957; Bucher, McGarrahan, Gould & Loud, 1959; De Matteis, 1968) suggested that the changes in the rate of cholesterol synthesis can be largely accounted for by changes in the rate of formation of mevalonic acid, probably through variations in the activity of HMG-CoA reductase. Direct assay of this enzyme has shown that its activity falls on feeding with cholesterol (Siperstein & Fagan, 1966; Linn, 1967) and on starvation (Regen, Riepertinger, Hamprecht & Lynen, 1966; Linn, 1967) and can be restored to normal values on re-feeding (Regen *et al.* 1966). The activity of the enzyme was not measured in the present work, but the finding that the conversion of acetate into acetoacetate was not increased by actinomycin D suggests that the stimulation of cholesterol synthesis occurs between HMG-CoA and mevalonate, i.e. at the level of the reductase.

It is not clear whether the observed changes in enzyme activity represent changes in the amount of the enzyme protein or variations in its catalytic ability. Frantz *et al.* (1954) have pointed out the inverse relationship between cholesterol concentration in the liver and rate of cholesterol synthesis. This observation is compatible with the idea that the activity of HMG-CoA reductase is controlled by the concentration of the end product of the pathway through an 'allosteric' type of inhibition, as suggested by Siperstein & Fagan (1964*a*). In the cholesterol-feeding experiments described in the present work (Table 6) the cholesterol-rich diet was withdrawn 24hr. before the labelling experiments and the animals were starved until they were killed: in those rats with a normal liver cholesterol concentration the incorporation of acetate into cholesterol was still depressed. This suggests that part at least of the feedback control exercised by dietary cholesterol may not result from a direct inhibition of the enzyme by excess of liver cholesterol, but from a more indirect mechanism, perhaps through feedback repression involving inhibition of the synthesis of the enzyme.

One possible mechanism by which actinomycin D might increase the activity of HMG-CoA reductase is by interfering with the feedback control exercised by cholesterol. Siperstein & Fagan (1964*b*) showed that hepatoma cells synthesize cholesterol at a faster rate than do normal liver cells and that the

*Abbreviation: HMG, β-hydroxy-β-methylglutaryl.

tumour tissue does not respond to the feedback control by excess of dietary cholesterol. In the present work, feeding with cholesterol almost completely suppressed the stimulation of cholesterol synthesis caused by actinomycin D and, when available in excess, dietary cholesterol was still able to activate the feedback control. Thus stimulation by actinomycin D cannot be due to a complete failure of the feedback mechanism. However, actinomycin D might stimulate cholesterol synthesis by decreasing the concentration of cholesterol at the site where the feedback control takes place. This might arise either by increased secretion of newly synthesized cholesterol into the blood or by accelerated conversion of cholesterol into bile salts.

Effect of inhibitors of protein synthesis and of adrenalectomy on the hepatic response to actinomycin D. The increased acetate incorporation into cholesterol caused by actinomycin D was abolished by DL-ethionine or by puromycin; this finding suggests a mechanism of regulation through changes in the amount of HMG-CoA reductase. Thus actinomycin D may stimulate cholesterol synthesis either by increasing the synthesis of this enzyme or by decreasing its rate of breakdown. Compatible with these possibilities are the findings that actinomycin D did not stimulate the incorporation of acetate into cholesterol when added to liver slices *in vitro*, and that the stimulation observed *in vivo* did not take place immediately, but gradually appeared after a short time lag.

A dose of actinomycin D (0·6mg./kg.) that stimulated the incorporation of acetate into cholesterol inhibited the incorporation of [6-^{14}C]-orotate into rapidly labelled liver RNA. This finding implies that any increased synthesis of HMG-CoA reductase brought about by the drug either does not require the formation of new messenger RNA or is mediated through the synthesis of a messenger RNA unusually resistant to actinomycin D. Other examples of stimulation of enzyme activity by actinomycin D have been reported (Pollock, 1963; Moog, 1964; Rosen, Raina, Milholland & Nichol, 1964; Garren, Howell, Tomkins & Crocco, 1964; Scarano, De Petrocellis & Augusti-Tocco, 1964; Goodwin & Sizer, 1965; Hilf, Michel, Silverstein & Bell, 1965; Drysdale & Munro, 1965; Della Corte & Stirpe, 1967; McCoy & Ebadi, 1967; Wing & Robinson, 1968), and in many of them (as in the present work) results have been obtained suggesting that the action of the drug is mediated through protein synthesis. The mechanism involved is not known, though several hypotheses have been put forward to try to reconcile these effects of the antibiotic with its property of inhibiting messenger RNA synthesis. Particularly attractive among these is the concept (Pollock, 1963) that increased synthesis of certain enzymes in response to relatively low doses of the antibiotic might result from preferential inhibition of the synthesis of the specific repressors concerned with restraining the formation of the respective enzymes. This interpretation could apply to the findings of the present work, though dietary cholesterol might have been expected to be less effective than it was in preventing the stimulation of cholesterol synthesis by actinomycin D: if cholesterol exercises its feedback control by repressing the formation of HMG-CoA reductase, this feedback control would probably require the presence of the repressor.

Actinomycin D has been shown to stimulate the secretion of corticosterone in the rat (Lippe & Szego, 1965); the finding that the increased incorporation of acetate into cholesterol caused by the drug was not completely abolished by adrenalectomy indicates that the stimulation of cholesterol synthesis is not entirely dependent on an increased activity of the adrenals. The same conclusion also applies to the stimulation by actinomycin D of the activity of other liver enzymes (Moog, 1964; Rosen *et al.* 1964; Garren *et al.* 1964; Della Corte & Stirpe, 1967).

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