Biphasic Effects of the Natural Estrogen 17β-Estradiol on Hepatic Cholesterol Metabolism in Intact Female Rats

Paolo Parini, Bo Angelin, Anneli Stavréus-Evers, Bo Freyschuss, Håkan Eriksson, Mats Rudling

Abstract—The protective influence of estrogens in cardiovascular disease is believed to be partly due to beneficial effects on cholesterol metabolism. Much of the experimental data are based on models in which synthetic estrogens have been used in pharmacological doses, and therefore, the physiological role of estrogens in cholesterol metabolism is uncertain. To evaluate this important issue, we performed experiments in intact female rats with use of the natural estrogen 17β-estradiol (E2) administered either subcutaneously or orally. After physiological doses of E2 (≤0.04 mg · kg⁻¹ · d⁻¹) were administered, plasma levels of high density lipoprotein (HDL) cholesterol and apolipoprotein (apo) A-I were increased. In the liver, 3-hydroxy-3-methylglutaryl coenzyme A reductase and cholesterol 7α-hydroxylase activities were increased, as well as cholesterol 7α-hydroxylase mRNA levels. These effects were abolished during treatment with higher doses of E2, whereas apo A-I mRNA increased in a dose-dependent way. After treatment with pharmacological doses of E2 (≥0.2 mg · kg⁻¹ · d⁻¹), the number of hepatic low density lipoprotein receptors increased and plasma cholesterol was reduced. These effects were similar after both oral and subcutaneous administration of E2. Our results show that the responses to E2 are biphasic: plasma HDL, apo A-I, and hepatic enzyme activities governing bile acid and cholesterol synthesis increased only at physiological doses of E2. At pharmacological doses of E2, hepatic low density lipoprotein receptors are stimulated and plasma cholesterol is reduced. Therefore, under physiological conditions, E2 exerts its major effects on hepatic cholesterol metabolism through mechanisms other than stimulation of low density lipoprotein receptor expression. (Arterioscler Thromb Vasc Biol. 2000;20:1817-1823.)

Key Words: apolipoprotein A-I ■ bile acids ■ lipoproteins ■ LDL receptors ■ estrogen receptors

Endogenous sex steroids are believed to protect premenopausal women against the development of coronary heart disease.1,2 Estrogens have been reported to have a number of potentially beneficial effects on lipoprotein metabolism, resulting in reduced LDL and increased HDL cholesterol in plasma.1-5 Attempts to identify the mechanisms by which endogenous estrogens modulate their effects in vivo are hampered by the difficulty of interpreting data from experimental models. Thus, the varying effects observed may be related to species, sex, and initial hormonal status (eg, postmenopausal or ovariectomized versus fertile). Differences in dose and efficiency between estrogen preparations, as well as the mode of administration (eg, oral versus parenteral), may also be important.

Much of our knowledge of estrogen’s effects on lipoprotein metabolism is based on studies in rats. In this species, the plasma total and HDL cholesterol levels are higher in females than in males.6,7 High doses of estrogens (1 to 5 mg · kg⁻¹ · d⁻¹ of ethynyl estradiol) reduce plasma cholesterol levels in male rats.8 This effect is in part caused by increased elimination of LDL from the plasma due to an increased hepatic LDL receptor (LDLR) expression.11,12 These responses to pharmacological doses of estrogens can be quenched by simultaneous administration of antiestrogens,13 indicating that the effects are estrogen receptor (ER) mediated. However, owing to the high doses used, it is still unclear whether these effects of estrogens are physiologically relevant.

During treatment with low doses of estrogens, HDL cholesterol is increased in the rat.14,15 Effects of low doses of 17β-estradiol (E2) on the activity of the rate-limiting enzyme in the conversion of cholesterol to bile acids, cholesterol 7α-hydroxylase (C7αOH), have also been described.16 The effects of pharmacological doses of E2 on this enzyme are less clear.17

The current investigation was performed to study the physiological relevance of the effects of estrogens on hepatic cholesterol metabolism in the intact female rat. Our results clearly show that physiological doses of E2 increase plasma HDL, apo A-I, and the enzymatic activities regulating the synthesis of bile acids and cholesterol. At pharmacological doses of E2, these responses are absent, whereas hepatic LDLRs are induced together with reduced plasma cholesterol.

Methods

Materials

E2 (17β-estradiol; catalog No. E-4) was from Sigma Chemical Co, and Alzet osmotic minipumps (model 2001) were from Alza Corp.

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All other reagents and chemicals were from previously described sources.  

**Animals and Experimental Procedure**

Altogether, 110 female Sprague-Dawley rats (250 to 300 g, 8 to 9 weeks old; B&K Universal, Stockholm, Sweden) were used in 3 separate experiments, for which each group consisted of 5 or 6 rats. Animals were kept under standardized conditions with free access to water and chow. The light cycle hours were between 6 AM and 6 PM. All studies were approved by the institutional Animal Care and Use Committee.

The drugs were dissolved in propylene glycol and administered subcutaneously under light ether anesthesia at 9 AM for 7 days. In the experiment in which E2 was infused subcutaneously, osmotic minipumps were implanted in the dorsal region. A small (5- to 10-mm) incision was made, and a subcutaneous tunnel was created with a pair of forceps. Minipumps were put in place and the skin was sutured. Controls were sham-operated with the same surgical procedure. The control groups in all experiments received vehicle. When the experiments were terminated (10 to 11 AM), the rats were anesthetized with ether. Blood was drawn by cardiac puncture and the animals killed by cervical dislocation. Livers were perfused with ice-cold PBS (140 mmol/L NaCl, 2.7 mmol/L KCl, and 9.5 mmol/L phosphate buffer, pH 7.4), removed, and immediately frozen in LN2.

In the experiment in which the activities of C7αOH and 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase were determined, a piece of fresh liver (~1 g) was taken immediately after the perfusion for preparation of microsomes as described below.

**Cholesterol Assays**

Cholesterol in the plasma and fast protein liquid chromatography (FPLC) fractions was assayed with the Boehringer Mannheim cholesterol assay kit (MPR 2 1 442 350) and a 5.2 mmol/L cholesterol standard from Merck. Size fractionation of lipoproteins by FPLC was performed on a Superose 6B column and using a previously described system.  

Equal volumes of plasma from individual animals were pooled (2.4 mL), and the density was adjusted to 1.21 g/mL with KBr. After ultracentrifugation at 100,000 × g for 48 hours, the supernatant was adjusted to 1.6 g/mL NaCl, 0.01% EDTA, and 0.02% NaN3, pH 7.3. One microliter of this solution, corresponding to 1.5 mL of plasma, was injected onto a 540×18-mm Superose 6B column after filtration through a Millipore 0.45×10−3-mm mixed cellulose ester filter. FPLC fractions of 2 mL were collected at a flow rate of 1 mL/min.

**SDS–Polyacrylamide Gel Electrophoresis (PAGE) Separation of Apolipoproteins**

From each group, 7.5 or 15 μL of ultracentrifuged plasma was added to loading buffer (final volume, 95 μL) and boiled for 5 minutes in the presence of 5% (vol/vol) 2-mercaptoethanol. Aliquots of 70 μL were loaded onto a 4% to 20% gradient SDS/polyacrylamide gel and separated for 4 hours at 45 mA. Gels were stained with Coomassie Blue. For reference, wide molecular-mass standards (Bio-Rad Laboratories) as well as human LDL and HDL were used. The bands corresponding to apo A-I were quantified by densitometry.

**Preparation of Hepatic Membranes and Ligand Blot Assay of LDLRs**

Liver membranes were prepared from pools of liver as described previously.  

Gels (6% SDS/polyacrylamide) were loaded with the indicated amount of membrane protein prepared from pooled samples of liver. Size markers were reduced with mercaptoethanol and boiled. Filters were incubated with 125I-Labeled rabbit β-migrating VLDL, as described previously.  

Filters were exposed on Dupont Cronex film. LDLR expression was quantified by using a Fujix Bio-imaging analyzer (BAS 2000, Fuji Photo Film Co). The values of the 120-kDa bands were expressed in arbitrary units after subtraction of filter background.

**Total Nucleic Acid Preparation**

Frozen liver specimens (0.2 g) were homogenized in 4 mL of SET buffer (1% [wt/vol] SDS, 10 mmol/L EDTA, and 20 mmol/L Tris-HCl, pH 7.5) with a Polytron (Kinematica, type PT 10/35, Kriens). The samples were subsequently sonicated on ice by 10 pulses in a Branson B 15 sonifier and digested with proteinase K (200 μg/mL) for 45 minutes at 45°C. Total nucleic acid was precipitated with ethanol after phenol-chloroform extraction, and the pellet was suspended in 300 μL of 0.2× SET buffer. The concentration of total nucleic acid in the samples was measured at 260 nm and assuming that 1 optical density unit was equivalent to 40 μg of total nucleic acid per milliliter. The DNA concentration was measured fluorometrically at 458 nm.

**Quantification of mRNA**

The mRNA levels for the LDLR, HMG-CoA reductase, and C7αOH were quantified by a solution hybridization titration assay with the use of mouse cRNA probes. The mRNA levels for apo A-I were quantified by using a rat cRNA probe corresponding to nucleotides 16 to 419 in the rat apo A-I cDNA. The probe was developed from a plasmid containing the full cDNA sequence of rat apo A-I, kindly provided by Dr Bart Staels, INSERM, Lille Cedex, France. Hybridization specificity was tested on total nucleic acid extracts from different rat organs (data not shown). The probe used for ER mRNA corresponds to nucleotides 1470 to 2062 of the mouse ER cDNA sequence, which encode the C-terminal half of the steroid-binding domain E and all of domain F. The slopes of the linear hybridization signals were calculated by the method of least squares and compared with the slope generated by the respective synthetic mRNA standard. Data are expressed as attomoles (10−18 moles) of mRNA per microgram of total nucleic acid.

**Preparation of Cytosol and ER Determination by Enzymatic Immunoassay**

All procedures were performed at 0°C to 4°C. Liver samples (0.5 to 1 g) were freeze-dried in glass centrifuge tubes for 92 hours and cut with a scalpel. Two milliliters of 0.4 mol/L KCl in TEM-SH buffer (10 mmol/L Tris-HCl, 1.5 mmol/L EDTA, 10 mmol/L sodium molybdate, and 1 mmol/L monothioglycerol, pH 7.4) was added to the tubes. The samples were extracted for 30 minutes, vortexed every 5 minutes, and then centrifuged at 7700g for 20 minutes. The supernatant was collected and the incubation and centrifugation procedures repeated with 1 mL of KCl in TEM-SH buffer. The supernatants were centrifuged again in a swing-out rotor at 23 000 × g for 65 minutes. Protein determination was performed according to Lowry et al. The protein concentration in samples was adjusted to 1 mg/mL, and an enzymatic immunoassay was performed by using a commercial kit and following the manufacturer’s instructions (Abbott Scandinavia AB).

**Activities of C7αOH and HMG-CoA Reductase**

Microsomes were prepared by differential ultracentrifugation of individual liver homogenates in the absence of fluoride as described previously. The activity of C7αOH was determined as the formation of 7α-hydroxycholesterol (pmol · min−1 · mg−1 protein) from endogenous microsomal cholesterol by using isotope dilution mass spectrometry. Microsomal HMG-CoA reductase activity was assessed by determining the conversion of [3H]HMG-CoA to mevalonate and expressed as picomoles formed per minute per milligram protein. The enzyme assays were carried out in duplicate.

**Statistics**

Data are presented as mean±SEM. The significance of differences between groups was tested by 1-way ANOVA, followed by planned comparison or post hoc comparisons of group means according to least significant difference methods (Statistica software, Stat Soft). To stabilize the variances, data were logarithmically transformed when a correlation between means and variances was found.
Results

In the first experiment, we wanted to determine the effect of increasing doses of E2, given as daily, single subcutaneous injections, on plasma lipoproteins in intact adult female rats. After 1 week of treatment with E2 at doses ranging from 0.01 to 4 mg·kg⁻¹·d⁻¹, it was found that plasma total cholesterol was reduced in animals treated with high doses (1 and 4 mg·kg⁻¹·d⁻¹) of E2 (not shown). An ∼30% reduction was present in animals receiving 1 mg·kg⁻¹·d⁻¹ (P<0.01), and a 55% reduction was seen in those receiving 4 mg·kg⁻¹·d⁻¹ (P<0.001). Separation of lipoproteins by FPLC showed that high doses of E2 (1 and 4 mg·kg⁻¹·d⁻¹) reduced cholesterol within both LDL and HDL fractions (Figure 1A). At low doses of E2, HDL cholesterol increased (Figure 1A). Analysis of apolipoproteins in ultracentrifuged plasma by SDS-PAGE separation showed that apo A-I levels were increased among animals receiving low-dose E2 and that this was most pronounced at a dose of 0.1 mg·kg⁻¹·d⁻¹ (Figure 1B).

Apolipoproteins in animals on the highest dose of E2 (4 mg·kg⁻¹·d⁻¹) could not be measured because of technical problems. Hepatic apo A-I mRNA levels increased dose-dependently (Figure 1C). Analysis of LDLR expression in liver membranes revealed a 4-fold stimulation at a dose of 1 mg·kg⁻¹·d⁻¹ and an ∼20-fold stimulation at 4 mg·kg⁻¹·d⁻¹ (Figure 2A). No LDLR stimulation occurred at lower doses of E2. The LDLR mRNA level was increased 2-fold at a dose of 1 mg·kg⁻¹·d⁻¹, and no further stimulation was seen at higher doses (Figure 2B). Assay of hepatic ER expression by enzyme immunoassay revealed a dose-dependent increase that was maximal at 1 mg·kg⁻¹·d⁻¹ of E2 (Figure 2C). Quantification of hepatic ER mRNA showed a dose-dependent reduction that was most pronounced in animals with the highest abundance of hepatic ERs (Figure 2D).

We then wanted to clarify whether the route of administration was important for the level of stimulation of hepatic LDLRs by E2. For this purpose, increasing doses (0.008 to 1 mg·kg⁻¹·d⁻¹) of E2 were given daily for 5 days to intact female rats by oral or subcutaneous route. Analysis of total plasma cholesterol showed reductions at high doses of E2, which were independent of the route of administration (Figure 3A). Hepatic LDLR expression was similarly increased in both groups of animals (not shown). Analysis of hepatic LDLR mRNA abundance revealed that oral administration of E2 increased the LDLR mRNA levels significantly by 2-fold at 1 mg·kg⁻¹·d⁻¹ only (Figure 3B). Subcutaneous injection of E2 resulted in a 2-fold increase of LDLR mRNA, not only at 1 mg·kg⁻¹·d⁻¹ but also at the lower dose of 0.2 mg·kg⁻¹·d⁻¹ (Figure 3B). Thus, there was no evidence of a more potent effect of oral compared with subcutaneous administration of E2.

Because single, daily bolus injections of E2 may result in nonphysiological oscillating plasma E2 levels, we then proceeded to determine whether a more physiological administration, viz, by continuous infusion with osmotic minipumps, could alter hepatic cholesterol metabolism at lower doses of E2. After 1 week of subcutaneous infusion with E2 into intact female rats, the animals were killed. Analysis of plasma total cholesterol showed an ∼35% reduction among rats receiving 0.2 mg·kg⁻¹·d⁻¹ of E2 and an ∼60% reduction in animals receiving 1 mg·kg⁻¹·d⁻¹. There were no reductions in plasma cholesterol in animals receiving lower doses of E2 (Figure 4A). When hepatic LDLR expression was analyzed by ligand blotting, a 2-fold increase was already found at a dose of 0.04 mg·kg⁻¹·d⁻¹ (Figure 4B). A 5-fold increase was observed in animals infused with 0.2 mg·kg⁻¹·d⁻¹ of E2, whereas a 4-fold increase was seen in animals receiving 1 mg·kg⁻¹·d⁻¹ (Figure 4B). Analysis of LDLR mRNA

![Figure 1. Effects of subcutaneous injection of increasing doses of E2 on plasma lipoprotein profile and apo A-I. Female rats (6 animals per group) received the indicated dose of E2 for 1 week. Animals were killed at 11 AM, 2 hours after the last E2 injection. A, Plasma lipoprotein profiles after separation by FPLC. Cholesterol concentration was determined on 2-mL fractions collected after separation of pooled plasma samples on Superose columns. * indicates vehicle; ○, 0.01 mg·kg⁻¹·d⁻¹ of E2; ■, 0.1 mg·kg⁻¹·d⁻¹ of E2; Δ, 1 mg·kg⁻¹·d⁻¹ of E2, and △, 4 mg·kg⁻¹·d⁻¹ of E2. B, Apo A-I levels in pooled ultra-centrifuged plasma. After SDS-PAGE separation, the bands corresponding to apo A-I were quantified by densitometry. Changes in lipoprotein profiles or apo A-I levels were not tested statistically. C, Apo A-I mRNA abundance in hepatic total nucleic acid extracts from each rat was measured by solution hybridization. Results are shown as mean±SEM.](image-url)
abundance showed increased levels only in animals receiving 1 mg kg\(^{-1}\) day\(^{-1}\) of E2 (Figure 4C).

We then determined the activities of the rate-limiting enzymes in bile acid and cholesterol synthesis, viz, C7\(\alpha\)OH and HMG-CoA reductase, in hepatic microsomes. The C7\(\alpha\)OH activity was stimulated at low doses of E2: a 65% stimulation was present in animals infused with 0.008 mg kg\(^{-1}\) day\(^{-1}\) of E2, and rats infused with 0.04 mg kg\(^{-1}\) day\(^{-1}\) showed a 45% increase (Figure 5A). No stimulation of C7\(\alpha\)OH activity occurred at higher doses of E2. The C7\(\alpha\)OH mRNA levels were increased by 60% in animals receiving the lowest dose of E2 (Figure 5B). At higher doses of E2, there were no significant increases in C7\(\alpha\)OH mRNA. The activity of HMG-CoA reductase in hepatic microsomes was increased by \(\approx\)75% in animals infused with 0.008 mg kg\(^{-1}\) day\(^{-1}\) E2 (Figure 5C). The induction of HMG-CoA reductase activity by E2 was maximal (\(\approx\)90% increase) at a dose of 0.04 mg kg\(^{-1}\) day\(^{-1}\) (Figure 5C). In animals infused with 0.2 mg kg\(^{-1}\) day\(^{-1}\) of E2, the microsomal activity of HMG-CoA reductase was increased by \(\approx\)45% (Figure 5C). There were no significant changes in HMG-CoA reductase mRNA levels at any dose (not shown).

**Discussion**

The present study has established that the metabolic response to E2 is biphasic in intact female rats. Thus, whereas some effects of estrogen, such as increased plasma apo A-I and HDL cholesterol levels as well as stimulated C7\(\alpha\)OH and HMG-CoA reductase activities, were already observed in the physiological dose range, the well-established effects of estrogens on hepatic LDLR and plasma cholesterol\(^8,11\) clearly required pharmacological doses.

Ovariectomized animals are commonly used as a model in which to study the effects of estrogens. However, it has been previously shown that ovariectomy results in a 2-fold increase in hepatic ER expression in rats.\(^29\) Thus, to avoid possible overinterpretation of the data, we chose to study the effects of different doses of estrogen on cholesterol and lipoprotein metabolism in intact female rats. In fertile females (humans as well as rats), there is a day-to-day variation in serum estrogen levels,\(^30,31\) and this variation throughout the cycle may be more important for biological responses than has been generally believed. The levels of serum E2 reached at the lower doses were clearly within this range, however.* Furthermore, at least in rats, ER and ER mRNA levels do not vary in the liver as much as in the uterus throughout the ovulatory cycle.\(^11\)
In agreement with previous work, the increase in LDLR expression obtained at high doses was also present at the mRNA level. This stimulation was much lower than that of LDLR protein, however. The cause for this discrepancy is unknown, but it may be due to an estrogen-induced increased efficiency of translation of LDLR mRNA. The fact that the hepatic ER number was increased by E2 treatment may also contribute to this phenomenon. In preliminary experiments in intact female rats (P.P. et al, unpublished observations), we found that the stimulatory effect of high-dose E2 on LDLR expression could be completely blocked by the concomitant administration of a highly selective antiestrogen, ICI-182,780. Because there was no effect of the antiestrogen when given alone, this finding indicates that the normal LDLR expression in the liver of female rats is not under control of endogenous estrogens, in consonance with our previous observations in the male rat.

In contrast to the findings for the LDLR, we could identify several important effects on hepatic lipoprotein and cholesterol metabolism that occurred at physiological doses of E2. Thus, the plasma apo A-I level increased by 3-fold at a low E2 dose (0.01 mg \( \cdot \) kg \( ^{-1} \cdot \) d \( ^{-1} \)) and was maximal at the 0.1 mg \( \cdot \) kg \( ^{-1} \cdot \) d \( ^{-1} \) dose of E2 (Figure 1B). The discrepancy of apo A-I at the highest dose is probably the consequence of pronounced stimulation of HDL and LDL clearance due to the very high expression of hepatic LDLRs at that dose. The discrepancy of these observations in intact female rats with the established increase in plasma apo A-I levels, which
occurs after estrogen therapy in human females.\textsuperscript{35} is probably due to species differences. The concentration of apo E, an efficient ligand for the LDLR, is indeed higher in rats than in humans, especially in HDL particles.\textsuperscript{36,37} An increase in LDLR expression in the liver of women treated with estrogen would therefore not affect the plasma levels of HDL cholesterol and apo A-I as it does in rats. The hepatic apo A-I mRNA levels were not significantly increased at the lowest dose but displayed a clear dose-dependent relationship throughout all E2 concentrations. These findings are in agreement with previous work in vitro\textsuperscript{38} and clearly also indicate that the variation of E2 levels within the physiological range influences plasma apo A-I and HDL cholesterol.

Thus, the higher HDL cholesterol levels in female compared with male rats\textsuperscript{7} are probably determined by their higher endogenous E2 levels. Accordingly, ovariectomized rats have lower plasma HDL cholesterol.\textsuperscript{7} Furthermore, the fact that HDL cholesterol is decreased in response to tamoxifen treatment in male rats (P.P. et al, unpublished observations, 2000) may argue in favor of a role for the ER in HDL cholesterol levels in this sex. Thus, estrogen “tonus” would seem to be an important factor in the normal regulation of HDL cholesterol levels in the rat.

The activities of C7\textalpha OH and HMG-CoA reductase were enhanced at low doses of E2. However, in contrast to the effects on apo A-I, there was no further stimulation at higher doses of E2. It is clear that the response patterns for C7\textalpha OH and LDLR are different. The activity of HMG-CoA reductase may in part be induced by an increased C7\textalpha OH activity at low E2 doses, whereas the reduced activity of the reductase at high E2 doses is probably related to the massive influx of lipoprotein cholesterol to the liver via the increased LDLRs. In a previous study from our group, the total cholesterol concentration in the livers of rats treated with high-dose estrogen was indeed shown to be increased.\textsuperscript{19} The fact that HMG-CoA reductase mRNA levels were not altered in these situations of varying enzyme activity may indicate that posttranscriptional mechanisms of regulation are predominant.\textsuperscript{39}

Finally, it should be realized that the stimulation of C7\textalpha OH activity observed here may not be a direct effect of E2. Low doses of E2 induce the release of pituitary growth hormone in the intact rat.\textsuperscript{40} Growth hormone administration has been shown to stimulate C7\textalpha OH activity and mRNA in intact rats.\textsuperscript{41,42} Therefore, the effects on C7\textalpha OH after E2 treatment may be secondary due to an enhanced growth hormone release. Further studies will be necessary to evaluate this interesting possibility.

In conclusion, our detailed studies on hepatic cholesterol metabolism in intact female rats treated with a natural estrogen have established that the responses to this steroid are biphasic. At low but not at high doses, C7\textalpha OH and HMG-CoA reductase activities are increased. At both low and high doses, apo A-I synthesis is increased in a progressive fashion, resulting in an increase in HDL cholesterol at low doses. At high (pharmacological) doses, LDLR expression is stimulated, resulting in drastic reductions of LDL and HDL cholesterol. The recognition of this response pattern is most important for understanding the normal regulation of plasma lipoproteins, because it implies that E2 normally exerts its major beneficial effects on hepatic cholesterol metabolism through mechanisms other than the stimulation of LDLR expression. Although the relevance of these findings to the human species has not been established, the finding that estrogen at physiological doses could induce hepatic responses and affect cholesterol metabolism in the liver suggests that not only the day-to-day variation in plasma estrogen levels but also the expression of the ER in the liver may play an important role in the physiological regulation of cholesterol metabolism. It will be important to verify whether the biphasic response observed in this study is similar for other estrogenic compounds, particularly in relation to their potential role in the treatment of diseases such as osteoporosis and atherosclerosis.\textsuperscript{1,2}

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