Studies on the Metabolism of Progesterone and Related Steroids *in vitro*

2. FACTORS INFLUENCING THE METABOLISM OF PREGNANE-3α:20α-DIOL BY RAT-LIVER HOMOGENATES, AND THE INVESTIGATION OF THE PRODUCTS OF METABOLISM

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In the first paper of this series (Grant & Marrian, 1950) experiments were described which showed that rat and rabbit liver preparations were able to metabolize pregnane-3α:20α-diol (pregnanediol) to unknown products *in vitro*. No metabolism occurred under anaerobic conditions unless a hydrogen acceptor (methylene blue) was added. The metabolism was uninfluenced by cyanide, and the evidence was compatible with the view that the agent in liver responsible for the metabolism of pregnanediol was a dehydrogenase. The possibility that more than one enzyme was involved was not excluded.

The work has now been extended with rat-liver homogenates, which have been employed in an investigation of factors influencing the metabolism of pregnanediol. The ultimate aim of these experiments was to obtain a system of optimum activity which could be used in an attempt to isolate products of metabolism in a large scale experiment.

It has been shown that the metabolism of pregnanediol by liver homogenates is increased by coenzyme I. In attempts to obtain the product or products of metabolism in large scale experiments pregnan-3α-ol-20-one was isolated, but in amounts only sufficient to account for approximately 10% of the metabolized pregnanediol.

EXPERIMENTAL

Materials and methods

Riboflavin and aneurin pyrophosphate were obtained from Roche Products Ltd.; adenosinetriphosphoric acid (ATP) was a gift from Dr H. Bergel, glutathione from Dr A. P. Meiklejohn and adenosinemonophosphoric acid (AMP) from Dr I. D. E. Storey. Coenzyme I (Co I) was prepared in the laboratory by the method of Le Page (1949), and its purity as determined spectrophotometrically (Le Page, 1947) was 71%. The weights and concentrations of Co I referred to are calculated as pure material unless otherwise stated. Pregnanediol dihemisuccinate (PDHS) was prepared as already described (Grant & Marrian, 1950). The difficulty previously experienced with PDHS tending to come out of solution was not found with later batches of this compound

using the more concentrated phosphate buffer described below.

The standard medium used throughout this investigation was as follows (final concentrations): 0·114 M-NaCl, 0·046 M-KCl, 0·001 M-MgSO$_4$.7H$_2$O and 0·056 M-potassium phosphate; pH 7·4. This medium, which will be referred to as the 'saline', differs from the calcium-free phosphate saline of Krebs & Eggleston (1940) used hitherto in the higher concentration of phosphate buffer. This increase in concentration was found desirable to maintain the pH during incubation with the concentration of homogenate used. No significant difference in results was observed when Na$^+$ in the saline was replaced by K$^+$ in order to obtain a medium more truly representative of intracellular fluid. As alteration in the concentration of K$^+$ appears to have a slightly adverse effect on the solubility of PDHS in saline, the Krebs-Eggleston type saline was retained although it admittedly does not resemble the inorganic pattern of intracellular fluid.

Homogenates were prepared from the livers of well fed female or male rats of the Wistar strain, approx. 1 year old. It was demonstrated in separate experiments (unpublished) that the sex of the animal did not influence the results. The rats were killed by dislocation of vertebrae in the neck. The livers were rapidly removed and chilled in crushed ice. After chopping roughly with scissors 3·5 g. liver were weighed into glass homogenizers (Potter & Elvehjem, 1936) standing in crushed ice, and ground with 6 ml. ice-cold water (unless otherwise stated) for approx. 1 min. with four passages of the pestle. The final homogenate was diluted to 10 ml. and kept at 0° until pipetted into incubation flasks.

The standard reaction mixture for 'incubation' experiments consisted of 2·5 ml. saline containing about 1 mg. pregnanediol as PDHS, and 1 ml. homogenate (giving a final concentration of 10% (wet wt./vol.) tissue). Unless otherwise stated, additions to this reaction mixture were made by weighing the substances concerned on a torsion balance, and adding the solid directly to the incubation flask. Freshly distilled pyruvic acid was diluted to suitable concentration, and carefully neutralized before adding to the saline.

In the quantitative experiments the procedure already described (Grant & Marrian, 1950) was followed. Incubations were in O$_2$ for 2 hr. at 37°. For 'controls' incubated reaction mixtures containing no PDHS were poured into 2·5 ml. PDHS saline and immediately worked up. 'Blank' values were found to be invariably low and very consistent. They were therefore not determined as a routine,

but were checked occasionally. A mean 'blank' value of 0·025 mg. 'apparent pregnanediol' was used to correct the values found in 'incubation' and 'control' experiments.

Decreased metabolism of pregnanediol observed when using rat-liver homogenates

The results of typical experiments using rat-liver homogenates are shown in Fig. 1 (bars *A* and *B*). The lower activity of liver homogenates in meta-bolizing pregnanediol will be evident from the fact that with these preparations over 80 % of the pregnanediol originally present was recovered, as
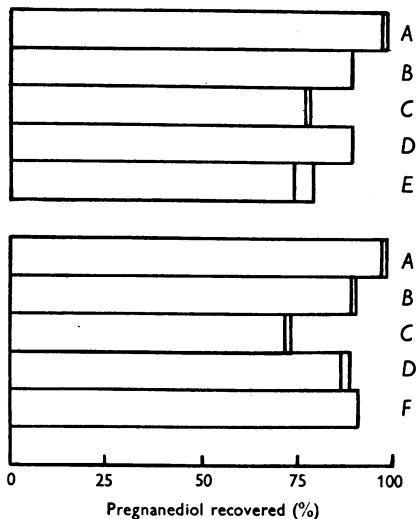

Fig. 1. Incubation of rat-liver homogenate with PDHS. Reaction mixture (*RM*) =about 1·6 mg. PDHS in 2·5 ml. saline +1 ml. homogenate. Pregnanediol recovered, *A*, from 'controls'; after incubation of the following: *B*, *RM*; *C*, *RM* +8 × 10⁻³M-nicotinamide; *D*, *RM* using homogenate pre-incubated 0·5 hr.; *E*, *RM* using homogenate pre-incubated 0·5 hr. with added nicotinamide (final concn. 8 × 10⁻³M); *F*, as *E* but nicotinamide added to homogenate after 0·5 hr. pre-incubation without nicotinamide.

In all figures two lines at ends of bars indicate two values, single lines duplicates.

compared with the recovery of only 50–60 % pregnanediol incubated under the same conditions with rat-liver slices (Grant & Marrian, 1950). A similar loss of activity on mincing liver has been observed in metabolic studies with oestrogens (Coppedge, Segaloff & Sarrett, 1950) and with testo-sterone (Sweat & Samuels, 1948), and has been attributed by these workers to the rapid destruction of Co I in preparations of broken cells from liver and other organs (Mann & Quastel, 1941). The pH of 7·4 used in the various liver-steroid metabolism experiments reported by others and used in the present investigation is close to the maxima of 7·2 (Spaulding

& Graham, 1947) and 7·5 (Handler & Klein, 1942) which have been reported for the nucleosidase concerned in the destruction of Co I. This de-struction is, however, inhibited by nicotinamide (Mann & Quastel, 1941; Handler & Klein, 1942).

Effect of preparing homogenates in nicotinamide. As it seemed likely that the reduced activity of rat-liver homogenates could be accounted for by the destruction of Co I in the homogenate, attempts were made to protect the Co I originally present in the liver by adding nicotinamide. Homogenates were prepared for each experiment from a single rat liver as follows: (*a*) in saline; (*b*) in saline containing nicotinamide sufficient to provide a concentration of 0·2 % in the final reaction mixture; (*c*) a portion of homogenate (*a*) was incubated 0·5 hr. at 37°, solid nicotinamide was then added to provide a final con-centration of 0·2 % (w/v) before use in PDHS experiments; (*d*) a portion of homogenate (*b*) was also incubated 0·5 hr. at 37° before use in the PDHS experiments. The results of experiments with these homogenates are shown in Fig. 1. It may be ob-served that there is an increase in metabolism of pregnanediol when using homogenates prepared in nicotinamide (bar *C*). Nicotinamide itself is not responsible for this increase since it has no effect when added to the homogenates which have been pre-incubated for 0·5 hr. (bar *F*). Homogenates which have been prepared in nicotinamide retain their higher activity on 0·5 hr. pre-incubation (bar *E*) supporting the view that the nicotinamide protects the Co I originally present. The observation that 0·5 hr. pre-incubation of homogenates pre-pared without nicotinamide did not further de-crease their activity in metabolizing pregnanediol (bar *D*) may find an explanation in the very rapid destruction of Co I, possibly before the pre-incubation started. Alternatively, if several pathways exist for the metabolism of pregnanediol by liver preparations and one or more is not Co I sensitive, it is conceiv-able that a limited direction of pregnanediol into such routes might mask the loss of activity due to decreasing concentration of Co I.

Effect of added Co I. The effect of various concen-trations of added Co I on the metabolism of preg-nanediol by rat-liver homogenates prepared in nicotinamide solution is shown in Fig. 2. From these results it appeared that rather high concentrations of Co I would be required to obtain the 50 % meta-bolism previously observed with liver slices. This might be due to stimulation of metabolism by an impurity in the Co I preparation, rather than by the Co I itself. Alternatively, the concentration of nicotinamide used may have been inadequate to protect the added Co I. AMP is the chief impurity in the Co I preparation (Le Page, 1949), but when this substance in concentration of 4 × 10⁻³M replaced Co I in the reaction mixture it had no effect on the

activity of the homogenate in metabolizing pregnanediol. In order to investigate the second possibility varying quantities of nicotinamide were
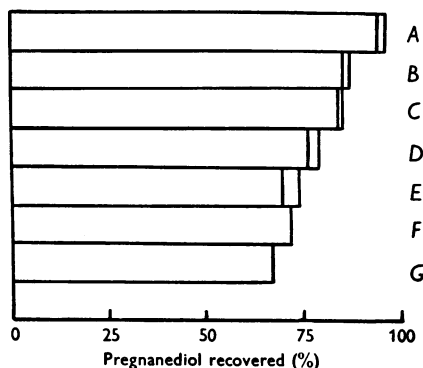

Fig. 2. Incubation of rat-liver homogenate with PDHS; influence of CoI on pregnanediol metabolism. Reaction mixture (RM) = about 1·6 mg. PDHS in 2·5 ml. saline + 1 ml. homogenate + 8×10^{-3} M-nicotinamide. Pregnanediol recovered is shown as follows: A, from 'controls'; B, after incubation of RM; after incubation of RM with CoI; C, 7×10^{-4} M-CoI; D, $1·3 \times 10^{-3}$ M-CoI; E, 2×10^{-3} M-CoI; F, $2·6 \times 10^{-3}$ M-CoI; G, $3·2 \times 10^{-3}$ M-CoI.
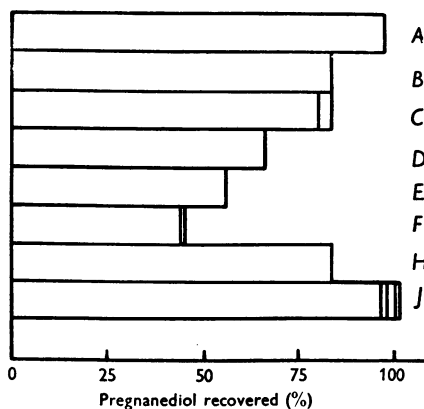

Fig. 3. Incubation of rat-liver homogenate with PDHS; protection of added CoI by nicotinamide. Reaction mixture (RM) = about 1·6 mg. PDHS in 2·5 ml. saline + 1 ml. homogenate. Pregnanediol recovered is shown as follows; A, from 'controls'; C, $RM + 8 \times 10^{-3}$ M-nicotinamide; D, $RM + 8 \times 10^{-3}$ M-nicotinamide + $1·5 \times 10^{-3}$ M-CoI; E, $RM + 4 \times 10^{-2}$ M-nicotinamide + $1·5 \times 10^{-3}$ M-CoI; F, $RM + 8 \times 10^{-2}$ M-nicotinamide + $1·5 \times 10^{-3}$ M-CoI; G, $RM + 0·12$ M-nicotinamide + $1·5 \times 10^{-3}$ M-CoI; H, $RM + 8 \times 10^{-2}$ M-nicotinamide; J, RM without homogenate + 8×10^{-2} M-nicotinamide + $1·5 \times 10^{-3}$ M-CoI.

added to the standard reaction mixture. When required, CoI was added (after the nicotinamide) to give a final concentration of $1·5 \times 10^{-3}$ M. It may be seen from the results shown in Fig. 3 that an

optimum effect was obtained using $1·5 \times 10^{-3}$ M-CoI and 8×10^{-2} M-nicotinamide (bar F).

This amount of nicotinamide had no significant effect in the absence of CoI (bar H). Incubation of PDHS with CoI and nicotinamide without liver homogenate did not result in any disappearance of pregnanediol (bar J).

Effect of other added substances. The following substances were tested individually in the standard reaction mixture containing $1·6 \times 10^{-2}$ M-nicotinamide and were found to be without effect on the metabolism of pregnanediol-ascorbic acid (7×10^{-9} M), glutathione (3×10^{-8} M), cocarboxylase (3×10^{-4} M), riboflavin (4×10^{-4} M), pyruvate (10^{-2} M), citrate (10^{-2} M), glucose (2×10^{-2} M), ATP (10^{-2} M).

The addition of 0·025 M-semicarbazide in an attempt to trap ketonic metabolic products was also without effect.

Investigation of the products of metabolism

Experiments on a larger scale were arranged to permit the attempts to isolate the product or products of metabolism of pregnanediol by rat-liver homogenates.

For this purpose pregnanediol was purified before conversion to PDHS by recrystallization from benzene and ethanol yielding a product which melted at 237–238°. (All melting points are corrected, and unless otherwise stated were determined on a Kofler-type apparatus; Klyne & Rankeillor, 1947.) The PDHS prepared in the usual way (Grant & Marrian, 1950) from this pregnanediol melted at 147–148°. (Found (material dried at 80° for 1 hr. *in vacuo* over P_2O_5): C, 67·1; H, 8·5. Calc. for $C_{29}H_{44}O_8$: C, 67·2; H, 8·5 %.)

The rat-liver homogenates were prepared in small batches in chilled glass homogenizers as described above. The Atomix Blender, with high-speed rotating blades, was not used for the preparation of the larger quantities of homogenate required since its use results in excessive frothing, the temperature is difficult to control and it has been reported by Stern & Bird (1949) and Lambden (1950) that the similar Waring Blendor is capable of inactivating certain enzymes.

The large scale experiment was conducted in five parts, a total of 500 mg. pregnanediol as PDHS being incubated with rat-liver homogenate.

The following is a description of a typical experiment. PDHS (163 mg. containing 100 mg. pregnanediol) was dissolved in 245 ml. saline containing 3·5 g. nicotinamide and 0·5 g. CoI. After checking that the pH was 7·4 the solution was diluted to 250 ml. Two 2·5 ml. volumes were removed for pregnanediol analyses, the remainder was cooled in ice and water.

Fresh rat liver (70 g.) was minced roughly with scissors, mixed and divided into two parts. One part was homogenized at 0° in seven 5 g. portions with seven 15 ml. volumes of the chilled PDHS saline. These homogenate reaction mixtures were pooled, thoroughly mixed with the bulk of the PDHS saline and distributed in ten 100 ml. conical flasks. The flasks were filled with O_2, stoppered, and shaken in a bath for 2 hr. at 37°.

The remaining 35 g. liver were treated in an identical fashion using saline which contained no PDHS and no CoI to provide a liver control experiment.

After incubation, the contents of the flasks were pooled, diluted to 300 ml. and two 3·0 ml. volumes were removed for pregnanediol analyses. The bulk of the mixture was poured into 1500 ml. cold dry acetone, thoroughly mixed in an Atomix Blender and filtered. The filter cake was washed with two 250 ml. volumes of acetone using the blender for efficient mixing. The filtrate was distilled under reduced pressure until the volume was 190 ml. The pH was found to be 7·0. This aqueous residue was transferred to large continuous liquid/liquid extractors with 50 ml. hot ethanol and

and 47 %. These figures indicate the extent of metabolism in each experiment.

The purity of the pregnanediol used in these experiments was checked in a pregnanediol control experiment as follows: 70 g. rat liver were homogenized and incubated in saline without PDHS, and an ether-soluble neutral fraction was prepared from the incubated material as already described. 200 mg. pregnanediol were then added and the mixture was subjected to the Girard separations and other procedures applied in the PDHS experiments.

The pooled ketonic and non-ketonic fractions from all experiments were brown gums. The weights of the various fractions are shown in Table 1.

Table 1. *Weights of non-ketonic and ketonic fractions isolated from large scale rat-liver homogenate experiments*

	Liver incubated (g.)	Pregnanediol incubated (g.)	Fractions isolated	
			Non-ketonic (g.)	Ketonic (g.)
PDHS experiment	175	0·5	4·175	0·208
Liver control	175	0	3·760	0·132
Pregnanediol control	70	0·2	0·964	0·074

extracted with ether (250 ml.) for 6 hr. adding 10 ml. ethanol to the aqueous phase after 2 and 4 hr. The pale-yellow ether extracts were washed twice with 50 ml. volumes 0·1 N-HCl, with 50 ml. 5 % (w/v) NaHCO₃ and finally with 2 × 50 ml. water. Emulsions formed during the early washings were broken by the addition of solid NaCl. The ether was evaporated to give 1·3 g. dark-brown gum in the PDHS experiment and 1·0 g. in the liver control experiment. Little significance was attached to these weights, since the gums appeared to retain solvent which was lost slowly in the vacuum desiccator. For the separation of ketonic material the ether-soluble neutral fractions, dried in a vacuum desiccator over CaCl₂ for 24 hr., were heated under reflux condenser for 1 hr. on a boiling-water bath with 0·5 g. Girard's Reagent T (Girard & Sandulesco, 1936), 1·5 g. glacial acetic acid and 10 ml. 95 % ethanol. After cooling, 88 ml. water, 30 g. crushed ice and 11·5 ml. 2 N-NaOH were added and the whole was extracted with 100 ml. and three 50 ml. volumes of ether. Troublesome emulsions usually formed during this extraction. The combined ether extracts were washed with 25 ml. 5 % NaHCO₃ and twice with 25 ml. water. A small amount of white solid which separated at the interface between ether and water during the washing was added to the ether phase. The washed ether was taken to dryness (non-ketonic fraction). 16 ml. conc. HCl were added to the ether-washed aqueous phase to hydrolyse Girard complexes. After standing overnight ketones were extracted with 100 ml. and three 50 ml. volumes of ether. The combined ether extracts were washed and evaporated as before to give the ketonic fraction.

On account of the troublesome emulsions formed during extraction of the non-ketonic fraction in all experiments it was thought that this fraction was likely to have retained ketonic material. It was therefore subjected to a second Girard separation in each case. Ketonic and non-ketonic fractions were subsequently pooled for further examination.

The quantities of pregnanediol recovered after incubation in the five experiments, calculated as percentages of the amount of pregnanediol originally present, were 44, 48, 49, 59

Each ketonic fraction was subjected to one further Girard separation yielding final ketonic fractions as follows: PDHS experiment, 57 mg.; liver control experiment, 13 mg.; pregnanediol control experiment, 12 mg.

Pregnan-3α-ol-20-one from the ketonic products of metabolism of pregnanediol by rat-liver homogenates

The final ketonic fraction in each case was dissolved in 10 ml. hexane and poured on to a column of 5 mm. diameter packed with 1 g. Al₂O₃ (Peter Spence and Co., Widnes, Type H, dried *in vacuo* at 100°, Activity II (Brockmann & Schodder, 1941)). The columns were eluted successively with four or five 10 ml. portions of each of the following anhydrous solvents: hexane, benzene, ether and acetone.

In the case of the ketonic fraction isolated from the PDHS experiment crystalline residues were obtained by evaporation of the benzene eluates (26 mg.). No crystalline material was obtained in the case of the liver control or pregnanediol control experiments.

An unsuccessful attempt was made to prepare a solid semicarbazone derivative from the gum (9 mg.) eluted by hexane in the case of the PDHS experiment.

The crystalline material from the benzene eluates (PDHS experiment) dissolved in 10 ml. hexane leaving a small amount of insoluble gum. The hexane solution was poured on to a column of Al₂O₃ prepared as already described, and the column was eluted successively with four 10 ml. portions of hexane, twenty-two 10 ml. portions of benzene, six 10 ml. portions of 3:1 benzene:ether and, finally, six 10 ml. portions of ether. Evaporation of the benzene eluates gave 21·5 mg. white crystalline solid which melted at 148–150°. After recrystallization from hexane and drying at 80° for 24 hr. *in vacuo* over P₂O₅ the substance melted at 148–149°. Mixed with authentic pregnan-3α-ol-20-one (m.p. 147–149°) the melting point was 147–148°. $[\alpha]_D^{14°} = +103\cdot6° \pm 0\cdot5$ in ethanol ($c = 0\cdot965$). Marrian & Gough (1946) reported $[\alpha]_D^{13°} = +107°$ in ethanol for authentic pregnan-3α-ol-20-one.

By treatment with semicarbazide hydrochloride and sodium acetate for 3 days at room temperature in aqueous

ethanolic solution, a product was obtained which after one crystallization from absolute ethanol and one from 80 % (v/v) aqueous ethanol melted at 249–251°; mixed with authentic pregnan-3α-ol-20-one semicarbazone (m.p. 248–250°) the melting point was 249–250°.

Investigation of non-ketonic fractions

Hexane-insoluble material was prepared from non-ketonic fractions by repeated leaching with boiling hexane and chilling the leachings for several hours in the refrigerator. In this way the non-ketonic fraction from the PDHS experiment and from the pregnanediol control experiment gave material which after recrystallization was identified as pregnane-3α:20α-diol. Crystalline pregnanediol (62 mg.) was obtained from the former experiment, 42 mg. from the latter. No hexane-insoluble material was obtained from the liver control experiment.

Hexane-soluble material in each experiment was dissolved in 50 ml. hexane and poured on to columns of 25 mm. diameter packed with 50 g. Al_2O_3. The columns were eluted successively with the following solvents: hexane, benzene, ether, acetone, ethanol and methanol. Cholesterol was isolated and identified from the acetone eluates from all experiments. The gums remaining after evaporation of the other solvents resisted all attempts at crystallization.

Thus no crystalline material except pregnanediol and cholesterol was obtained from the non-ketonic fractions.

DISCUSSION

The evidence presented in this paper shows that Co I is required by the enzyme system or systems involved in the *in vitro* metabolism of pregnanediol by rat liver.

In the investigation of the products of metabolism approximately half of the pregnanediol incubated with the liver was metabolized in some way. Evidence was presented in a previous paper (Grant & Marrian, 1950) to show that the pregnanediol which disappears cannot be accounted for by the formation of conjugated substances, nor can the pregnanediol which disappears be accounted for by absorption on the relatively large weight of liver tissue, since the treatment with potassium hydroxide at 100° before extraction of the pregnanediol dissolves the tissue in addition to hydrolysing any unchanged dihemisuccinate, and added preg-

nanediol has always been recovered in good yield from these experiments carried out in nitrogen in place of oxygen. In the experiments now reported a little over 10 % of the pregnanediol metabolized was accounted for as crystalline ketonic material, which appeared to consist mainly of pregnan-3α-ol-20-one. No evidence was obtained regarding the fate of the main part of the pregnanediol which disappeared.

Sweat, Samuels & Lumry (1950) have prepared an enzyme from steer liver which catalyses the oxidation of testosterone to androst-4-ene-3:17-dione. Co I was found to be necessary as a hydrogen acceptor in the system but marked reduction of the cytochrome *c* concentrations of the preparation did not affect its activity. The presence of this enzyme in the livers of a variety of species, including the rat, has also been demonstrated (Samuels, Sweat, Levedahl, Pottner & Helmreich, 1950). The possibility that the same enzyme may be involved in the oxidation of pregnane-3α:20α-diol to pregnan-3α-ol-20-one cannot be excluded, since Samuels and his co-workers have not investigated the effect of their enzyme on 20-hydroxy steroids (Samuels, 1951). Both the 'testosterone' and the 'pregnanediol' enzyme require Co I, and both appear to be capable of functioning in systems independent of cytochrome *c*.

SUMMARY

1. It has been shown that coenzyme I is required by an enzyme system or systems in rat-liver homogenates, which are able to metabolize pregnane-3α:20α-diol.

2. In a large scale experiment 50 % of the pregnane-3α:20α-diol incubated with rat-liver homogenate was metabolized. Of this amount 10 % was recovered as crystalline pregnan-3α-ol-20-one. No other crystalline products of the metabolism of pregnanediol were obtained.

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