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The Effect of Oestrogens and Chemically Related Compounds on the Respiration of Yeast and on Oxidative Phosphorylation

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It was shown by Shacter (1953*a*) that 4:4'-dihydroxy-$\alpha\beta$-diethylstilbene (stilboestrol) has an action on the metabolism of respiring yeast cells which shows a similarity to that of 2:4-dinitrophenol (DNP) on these cells. With increasing concentration there was at first a slight stimulation of yeast respiration, but as the stilboestrol concentration reached the value of 10^{-4}M the rate of oxygen uptake suddenly increased greatly, to a maximum value of 14 times that of the control, at 2×10^{-4}M, after which it began to fall until an inhibition was observed at concentrations above 10^{-3}M. These experiments were performed with yeast which had been washed and aerated in order to decrease the amount of oxidizable substrate in the cells. Stilboestrol was found to cause about three times the increase in respiration that was produced by 2:4-dinitrophenol. When yeast was respiring in the presence of glucose, low concentrations of stilboestrol did not increase the rate of oxygen uptake, and, in fact, as the concentration of stilboestrol was raised, respiration was gradually inhibited until it ceased completely at a concentration of stilboestrol of 10^{-4}M. Shacter (1953*b*) also found that *p*-chloromercuribenzoate, at high concentrations, stimulates the endogenous respiration of yeast in a rather similar manner. It was therefore suggested that 2:4-dinitrophenol, sulphydryl reagents and stilboestrol might affect yeast metabolism by similar mechanisms.

The following experiments were carried out in order to determine to what extent this type of action is limited to substances having oestrogenic activity, in a group of compounds which have a close chemical relationship to stilboestrol. Some powerful natural oestrogens were included.

In addition an attempt has been made to determine the effect of a similar range of compounds on hexokinase and myosin adenosine triphosphatase (ATPase) activity and on the oxidative phosphorylation in cyclophorase preparations.

MATERIALS AND METHODS

Adenosine triphosphate (ATP). The disodium salt was purchased from Sigma Chemical Co., U.S.A.

Stilboestrol and hexoestrol. These were commercial products; the other oestrogenic and related compounds were kindly given by Mr W. Lawson.

Phosphate buffer, pH 7·4. This consisted of 8 vol. of 0·1M-K_2HPO_4 and 2 vol. of 0·1M-KH_2PO_4.

Inorganic phosphate. This was determined by the method of Fiske & Subbarow (1925), after deproteinizing with trichloroacetic acid, as described below.

Yeast suspension. Fresh baker's yeast (Distillers' Co. Ltd.) was washed three times with distilled water, suspended in approximately 100 vol. of distilled water and aerated overnight at room temperature, in order to decrease the oxidizable substrates in the yeast cells to a low value.

Myosin. This was prepared from rat skeletal muscle by the method described by Dickens & Glock (1951). After four precipitations by dilution with de-ionized water, the myosin was dissolved in 0·5M-KCl. (Dry wt. of myosin, 0·5 mg./ml.)

Hexokinase. This was prepared from baker's yeast according to the method of Berger, Slein, Colowick & Cori (1946). The method was followed as far as the second ethanol precipitation, the fraction obtained between 29 and 45% ethanol being retained. This was dissolved in 1% glucose solution and stored at −20°.

Effect of various compounds on yeast respiration. This was determined at 30° in an atmosphere of air by incubating 1 ml. of the yeast suspension (see above) (approx. 20 mg. dry weight of yeast) with 0·2 ml. of 0·67M-KH_2PO_4 and distilled water to a final volume of 2 ml. in Warburg flasks,

and measuring oxygen uptake by the Warburg technique in the presence or absence of stilboestrol and chemically related compounds. These compounds were dissolved in the minimum amount of 0·01 N-NaOH and added (0·2 ml.) from the side arm of the Warburg vessel. An equivalent amount of NaOH was added to the controls without stilboestrol. The resulting pH of the vessel contents was 5·2 (glass electrode). The centre well of the vessels contained 0·2 ml. of 20% KOH as CO_2 absorbent.

Cyclophorase preparations from rat liver were carried out by the method of Green, Loomis & Auerbach (1948). The tissue dispersion was prepared in an M.S.E. blender.

Hexokinase activity. This was determined manometrically by measurement of acid production due to the transfer of phosphate from ATP, by the method of Berger *et al.* (1946).

Measurement of oxidative phosphorylation. The cyclophorase suspension was used within 0·5 hr. of its preparation for the determination of oxygen uptake and disappearance of inorganic phosphate, by the method of Judah & Williams-Ashman (1951). For each determination four Warburg flasks were used. Additions were as shown in Table 2. The cyclophorase preparation was added last, and the flasks were quickly placed in a bath at 30° and allowed to equilibrate for 2–3 min. In order to determine the amount of inorganic phosphate present at the beginning of the experiment, two flasks were deproteinized immediately by addition of 0·5 ml. of 70% (w/v) trichloroacetic acid from the side arm, and the oxygen uptake of the other two flasks was measured for 10 min. These were then also deproteinized, the contents of all four flasks were centrifuged and samples of the supernatant fluids were used for estimations of inorganic phosphate by the method of Fiske & Subbarow (1925).

ATPase activity of myosin. A solution containing 0·2 ml. of myosin in 0·5 M-KCl (0·5 mg. protein/ml.) was incubated in centrifuge tubes with 0·6 ml. of 0·1 M glycylglycine buffer, pH 8·9; 0·1 ml. of 0·1 M-$CaCl_2$, water to a final volume of 2 ml. and either stilboestrol or related compounds, dissolved in dilute NaOH, or an equivalent amount of NaOH without any addition in the control experiments. After 2–3 min. in a bath at 30°, 0·4 ml. of 0·013 M ATP was added to start the reaction. Trichloroacetic acid (0·3 ml. of 70% w/v) was added after 15 min. and, after centrifuging to remove the precipitated protein, inorganic phosphate was estimated in 1 ml. of the supernatant.

RESULTS

Effect of stilboestrol and chemically related compounds on the respiration of yeast

The effect on the respiration of a suspension of impoverished yeast cells, of stilboestrol and chemically related oestrogenic and non-oestrogenic compounds, as well as oestrogens of the steroid type, was determined by measuring oxygen uptake by the Warburg technique, as described under Methods. Typical results are shown in Fig. 1. In each case all the effective compounds tested increased the respiration over only a very narrow range of concentrations, starting at approximately 10^{-4} M, and at concentrations above 10^{-3} M the oxygen consumption was depressed. From Table 1 it can be seen that there is no clear relationship between the biological activity of the compounds and their effect in increasing respiration. It appears that for activity on respiration the molecule must possess two unsubstituted phenolic groups and that the carbon atoms joining these two rings must have an alkyl side chain without polar substituents such as –OH or $-CO_2H$. None of the steroids tried was effective in increasing oxygen uptake.

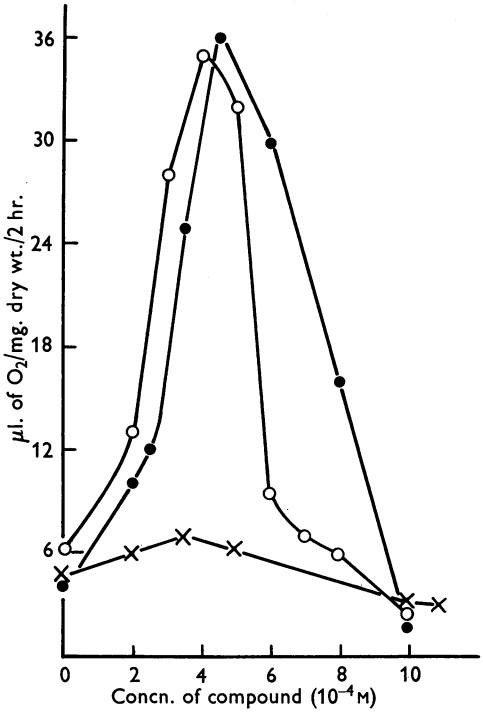

Fig. 1. Effect of stilboestrol and 4:4′-dihydroxystilbene on the respiration of yeast cells. ●, Stilboestrol: oestrogenic rat dose 0·35 μg.; ×, 4:4′-dihydroxystilbene: oestrogenic rat dose 5–10 mg.; ○, 1:2-di-(4′-hydroxyphenyl)*cyclo*hexane: oestrogenic rat dose >10 mg. Conditions similar to those shown in Table 1.

The increase in respiration of the yeast cells caused by the effective substances is maintained at a steady level for the whole period of the experiment, usually 2 hr. (Fig. 2).

For the complete oxidation to CO_2 and water of 2·0 ml. of stilboestrol solution at a concentration of $4·5 \times 10^{-4}$ M, this being the concentration at which the effect on respiration of yeast cells is greatest, 443 μl. of O_2 would be required. The value of the oxygen uptake, after 2 hr., of yeast cells respiring in the presence of $4·5 \times 10^{-4}$ M stilboestrol is far in

Table 1. *Effect of stilboestrol and related compounds on yeast respiration*

1 ml. of yeast cell suspension (approx. 20 mg. dry wt.), 0·2 ml. of 0·67 M-KH_2PO_4, water to a total volume of 2 ml. Compounds (in dil. NaOH) added from side arm. 0·2 ml. of 20% KOH in centre well; gas phase, air; 30°. The respiration rate for the yeast without addition was 4·6 μl. O_2/mg. dry wt./2 hr. (cf. Fig. 1).

Compounds added	Oestrogenic dose for the rat	Increase in respiration (μl. O_2/mg. dry wt./2 hr.)	Optimum concn. of compound added* (M $\times 10^{-4}$)
2:3-Di-(4-hydroxyphenyl)-2-methylpentane	2 μg.	38	5
Diethyldi-(4-hydroxyphenyl)methane	10 mg.	38	9
Stilboestrol	0·35 μg.	32	4·5
1:2-Di-(4-hydroxyphenyl)*cyclo*hexane	> 10 mg.	28	4
Hexoestrol	0·2 μg.	25	8
Di-(4-hydroxyphenyl)dimethylethane	50–60 μg.	23	8
*iso*Hexoestrol	0·5 mg.	21	5
$\alpha\beta$-Diethyl-2:2′-dihydroxystilbene	1 mg.	8	7
4:4′-Dihydroxystilbene	5–10 mg.	2	3·5
Disodium stilboestrol-*O*-*O*-diacetate	10 mg.	0	—
Dihydroxyhexoestrol	> 10 mg.	0	—
Di-(4-hydroxyphenyl)adipic acid	> 10 mg.	0	—
$\alpha\beta$-Dimethylstilboestrol monomethyl ether	1 mg.	0	—
4:4′-Dihydroxydibenzyl	10 mg.	0	—
Quinol	—	0	—
Phenol	—	0	—
Oestrone	0·8 μg.	0	—
Oestradiol	0·2–0·3 μg.	0	—
Bisdehydrodoisynolic acid	1 μg.	2	2

* These figures refer to the concentration at which the maximum effect on respiration is obtained.

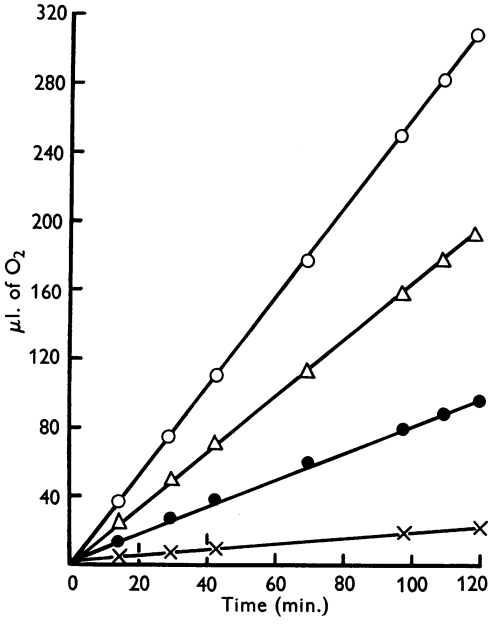

Fig. 2. Effect of stilboestrol on the rate of the oxygen uptake of yeast cells. ×, Control; ●, 2×10^{-4} M stilboestrol; ○, $4{\cdot}5 \times 10^{-4}$ M stilboestrol; △, 6×10^{-4} M stilboestrol. Conditions similar to those shown in Table 1 (dry wt. of yeast, approx. 10 mg.).

excess of this figure, however (Table 1). Hence it can be concluded that the increase in oxygen uptake is not due to the oxidation of stilboestrol by yeast enzymes. Experiments to measure the loss of phenolic groups of stilboestrol during incubation also showed little change.

The effect of stilboestrol on the exogenous respiration of yeast cells, in the presence of substrate, was also investigated. In the presence of acetate or ethanol as substrate, oxygen uptake was inhibited by stilboestrol at concentrations at which it increased the respiration of impoverished yeast, but with glutamate, which was oxidized only slowly by this yeast suspension, stilboestrol had no effect.

Effect on oxidative phosphorylation

DNP has been shown to uncouple oxidative phosphorylation. In concentrations of 5×10^{-5}–2×10^{-4} M, DNP was found to prevent the uptake of inorganic phosphate without affecting, or with slight stimulation of, the oxidation of glutamate by a rabbit-kidney homogenate (Loomis & Lipmann, 1948).

In order to determine whether stilboestrol and related compounds have a similar effect on oxidative phosphorylation, cyclophorase preparations from rat liver were incubated with some of these

Table 2. *Effect of stilboestrol and related compounds on oxidative phosphorylation in a cyclophorase preparation*

0·3 ml. of 0·01 M adenosine 5′-P, 0·1 ml. of 0·2 M-$MgSO_4$, 0·3 ml. of 0·2 M phosphate buffer, pH 7·4 ($KH_2PO_4 + K_2HPO_4$), 0·3 ml. of 0·1 M sodium glutamate, 0·3 ml. of 0·26 M fructose, 0·5 ml. of hexokinase, 1 ml. of cyclophorase preparation (equivalent to 2 g. fresh wt. liver), 0·1 ml. of 0·39 M-NaF, ±0·1 ml. of stilboestrol, were incubated in main compartment of Warburg vessels, 0·5 ml. of 70% trichloroacetic acid in side arm. Gas phase, air; 30°; 0·2 ml. of 20% KOH in centre well. Duration of experiment, 10 min.

Addition	μg.-atoms O_2	μg.-atoms P	P/O ratio	Inhibition of P/O ratio (%)
None	11·0	23·5	2·1	—
4×10^{-4} M stilboestrol	8·2	0	0	100
3×10^{-4} M stilboestrol	8·8	8·1	0·92	56
10^{-4} M stilboestrol	8·0	10·7	1·36	35
5×10^{-5} M stilboestrol	10·8	18·4	1·7	19
None	10·5	17·9	1·7	—
2×10^{-4} M hexoestrol	9·5	2·8	0·29	83
10^{-4} M hexoestrol	11·2	10·5	0·93	45
None	9·5	19·9	2·1	—
2×10^{-4} M dihydroxystilbene	10·8	17·9	1·6	24
None	11·0	17·5	1·6	—
4×10^{-4} M dihydroxyhexoestrol	10·9	16·6	1·5	0

compounds in the presence of glutamate as substrate (Table 2). Oxygen uptake and disappearance of inorganic phosphate were measured, as described under Methods. It can be seen that both stilboestrol and hexoestrol, as well as increasing the respiration of yeast, inhibit the phosphorylation coupled to the oxidation of glutamate. Dihydroxystilbene also does so to a lesser degree, and dihydroxyhexoestrol has no effect on yeast respiration or on oxidative phosphorylation.

The lowering of the P/O quotient was not due to the inhibition by these compounds of the yeast hexokinase which was added to the above system, as is shown in Table 3.

Table 3. *Effect of stilboestrol on hexokinase activity*

0·5 ml. of hexokinase; 0·5 ml. of 0·054 M-$NaHCO_3$; 0·1 ml. of 0·5 M glucose; 0·2 ml. of M-NaF; ±0·1 ml. of stilboestrol; water to total of 2·5 ml. in main compartment. 0·4 ml. of 0·02 M-ATP; 0·05 ml. of 0·1 M-$NaHCO_3$; 0·05 ml. of 0·2 M-$MgCl_2$ added from side arm. Gas phase, $N_2 + CO_2$ (95:5); 30°.

Conditions	μl. CO_2/ first 20 min.
No stilboestrol	130
5×10^{-4} M stilboestrol	129
2×10^{-3} M stilboestrol	130
4×10^{-3} M stilboestrol	130
No ATP	0
No hexokinase	0

Table 4. *Effect of stilboestrol and related compounds on the ATPase activity of myosin*

0·5 ml. of myosin (0·5 mg. protein/ml.); 0·6 ml. of 0·1 M glycylglycine buffer, pH 8·9; 0·1 ml. of 0·1 M-$CaCl_2$; 0·4 ml. of 0·013 M ATP; stilboestrol and other compounds dissolved in NaOH, water to final volume of 2 ml. Incubated 15 min. at 30°. The value at zero time has been subtracted in each case (average value 17 μg. P/ml. TCA supernatant).

Compound added	Concn. (M)	Total μg. P liberated/15 min.	Inhibition (%)
None	—	82	—
Stilboestrol	10^{-7}	82	0
	10^{-6}	82	0
	5×10^{-5}	81	1
	10^{-4}	74	10
	4×10^{-4}	40	51
	7×10^{-4}	32	61
None	—	56	—
Dihydroxyhexoestrol	4×10^{-4}	56	0
None	—	82	—
Diethyldi-(4-hydroxyphenyl)methane	10^{-4}	77	6
	4×10^{-4}	53	36

Effect on ATPase

The effect of stilboestrol and related compounds on the ATPase activity of a myosin preparation was determined by measuring the amount of inorganic phosphate liberated on incubation of the enzyme with ATP in the presence of 5×10^{-3}M-$CaCl_2$ and buffer at pH 8·9. In contrast to the effect of DNP, which in suitable concentrations enhances ATPase activity (Lardy & Wellman, 1953), stilboestrol and diethyldi-(4-hydroxyphenyl)-methane were found to inhibit the ATPase activity, whereas dihydroxyhexoestrol, which was shown above not to uncouple oxidative phosphorylation in liver preparations, had no action (Table 4).

Attempts to reverse the inhibition by stilboestrol of myosin ATPase by incubating the enzyme with cysteine (10^{-2}M) under anaerobic conditions in Thunberg tubes for 15 min. at 30° were unsuccessful and no reactivation was obtained.

DISCUSSION

The effect of increasing the endogenous respiration of yeast cells at low concentrations and inhibiting at higher ones was observed in these experiments to be caused by many compounds which are chemically related to stilboestrol. Several of these substances are powerful oestrogens, although some of them, such as 1:2-di(4′-hydroxyphenyl)*cyclo*hexane, which are biologically inactive, also increase the oxygen uptake of yeast cells to the same extent as the oestrogenic compounds. Of the compounds investigated so far, none that lack this ability to increase respiration is oestrogenically active, with the exception of $\alpha\beta$-dimethylstilboestrol monomethyl ether, which has only very slight oestrogenic activity. It can be concluded that the effect on yeast respiration by the compounds shown in Table 1 is not clearly related to their biological activity, but appears to depend on the presence in the molecule of two unsubstituted phenolic groups and non-polar alkyl side chains on the carbon atoms joining the two benzene rings. Neither of the two naturally occurring oestrogens, oestrone and oestradiol, was found to increase the rate of oxygen uptake of yeast cells at any concentration tested.

Hochster & Quastel (1949) observed the inhibitory action of stilboestrol on various dehydrogenase systems, and this they attributed to its role, in the form of a quinone, as a competitive hydrogen carrier. By analogy, Shacter (1953*a*) suggested that the inhibitory effect on yeast respiration may also be due to the action of stilboestrol as a competitive hydrogen carrier. This, however, is unlikely according to the experiments shown in Table 1, since compounds, such as diethyldi-(4-hydroxyphenyl)methane, which have the same effect as stilboestrol on yeast respiration are incapable of forming *para*-quinones without rupture of the molecule, as the carbon atom joining the two benzene rings is fully saturated by alkyl groups, there being thus no possibility of double-bond formation.

The action of stilboestrol and related compounds was found to be very similar to that of DNP, not only on yeast respiration, but also on oxidative phosphorylation in a cyclophorase system, the latter being completely inhibited by 4×10^{-4}M stilboestrol. It has been suggested (Hunter, 1951) that this uncoupling of oxidation from phosphorylation may be due to the breakdown of ATP by ATPase, the activity of which is enhanced by DNP. Stilboestrol, on the other hand, inhibited ATPase activity of myosin. Some preliminary experiments on the effect of stilboestrol on this enzyme indicate that this inhibition is probably non-competitive, as it seems to be independent of substrate concentration. As it was not possible to reverse the inhibition of myosin ATPase by means of cysteine, it is unlikely that the inhibition is due to the interaction of stilboestrol with the sulphydryl groups of the enzyme, unless the combination is unusually firm.

Since certain thyroid hormones also uncouple phosphorylations in mitochondrial preparations (cf. Klemperer, 1955) and since the more rapidly acting acetic acid analogues of thyroxine and triiodothyronine cause an increase in kidney-slice respiration *in vitro* (Thibault & Pitt-Rivers, 1955), it is proposed now to extend this study to include the effects of these compounds on the systems studied in this paper.

SUMMARY

1. The effect on the endogenous respiration of yeast cells of stilboestrol, a group of compounds chemically related to it, and some naturally occurring oestrogens, has been investigated.

2. It has been shown that the increase in yeast-cell respiration caused by stilboestrol and many chemically related compounds is not only a property of the biologically active substances, but depends on the chemical structure of the compounds. The natural oestrogens, oestrone and oestradiol, had no effect on the rate of oxygen uptake of yeast cells.

3. When yeast cells were respiring in the presence of acetate or ethanol, the oxygen uptake was inhibited by stilboestrol. In the presence of glutamate, stilboestrol had no effect on yeast respiration.

4. The substances which are able to increase yeast-cell respiration have been shown to uncouple oxidation from phosphorylation in cyclophorase preparations.

5. Myosin adenosine triphosphatase is strongly inhibited by stilboestrol and related compounds. This inhibition is probably of the non-competitive type.

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The Excretion of Amino Acids by the Human

A QUANTITATIVE STUDY WITH ION-EXCHANGE CHROMATOGRAPHY

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Application of the simple, highly specific method of paper chromatography (Consden, Gordon & Martin, 1944) to urine analysis (Dent, 1946) confirmed that certain amino acids were excreted in small amounts in the urine of healthy subjects. Definite patterns of excretion have been later demonstrated, characteristic of the individual and varying but little with exogenous changes (Dent & Harris, 1951; Dent, 1952). Stein (1953), using chromatography on columns of ion-exchange resins, has now reported accurate values for twenty-one urinary amino acids.

In the present study an excessive excretion of some or many of the amino acids in amounts much greater than normal is taken as a definition of amino aciduria. Certain patients show amino aciduria together with characteristic, but apparently unrelated, symptoms and biochemical changes. Such disorders of amino acid output are thought to occur mainly as a result of two distinct mechanisms, the 'renal' mechanism, when the plasma levels are normal and the 'overflow' mechanism when a rise in plasma level is the immediate cause of the increased excretion (Dent, 1954).

Much of the evidence for this hypothesis rests on qualitative experiments, using chiefly paper chromatography. The more quantitative data have been obtained by methods of an insufficiently specific kind, such as α-amino nitrogen determinations, and have given rise to controversy. Thus in the Fanconi syndrome conflicting opinions are held: Dent (1947, 1954) has reported normal plasma levels, but Bickel & Smellie (1952) and Woolf (1951) have reported levels raised 50–100% above the normal range, so that only Dent considers this amino aciduria to be renal.

Of the general methods available for the quantitative determination of amino acids, paper chromatography is subject to large errors with biological fluids. Microbiological assay is limited by several important criteria. For example, only an amino acid for which a known organism has a specific growth requirement can be assayed, the values obtained being liable to error if other compounds can be substituted for the test substance. On the other hand, the quantitative method of ion-exchange chromatography (Moore & Stein, 1951; Stein, 1953) is accurate within the limits of experimental error, can assay all the amino acids present, is unaffected by combined forms or homologues of the amino acids and facilitates the identification of previously unknown constituents. The experimental conditions used do not permit separation of optical isomers, if present, but this is probably unimportant except in feeding experiments with unnatural isomers, which have higher renal clearances than the natural isomers (Bonetti & Dent, 1954). The ion-exchange method, although time-consuming, permits a re-examination of the

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