Biochem. J. (1966) 101, 441

The Hydrating Effect of Lathyrogenic Compounds on Chick-Embryo Cartilage *in vivo*

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(*Received* 21 *April* 1966)

1. Treatment of the chick embryo with the lathyrus factor, β-aminopropionitrile, produces inhibition of polymerization of newly synthesized collagen at the inter- and intra-molecular level (Levene & Gross, 1959; Martin, Gross, Piez & Lewis, 1961); this is manifest as an increase in the fragility and solubility of collagen from the connective tissues. 2. This treatment simultaneously produced increased hydration of the tissues, particularly noticeable in the swollen long-bone cartilages. 3. This increased hydration of cartilage was found to result from treatment with any of the known lathyrogenic compounds, but not with any of the structurally related but non-lathyrogenic analogues. 4. The hydration effect paralleled the collagen solubility effect, both being dosage-dependent at the lower levels. 5. The hydration effect could not be reversed by treatment *in vivo* with either pyridoxal or glyceraldehyde, or by Benadryl, an antihistamine that inhibits cell water movement. 6. The possible causes of this effect have been considered in the light of the known properties of cartilage.

Treatment of experimental animals with the lathyrus factor, BAPN,† produces, in addition to the collagen defect, an increase in hydration of various tissues, such as cartilage (Borle, Karnovsky & Nichols, 1959), skin and bone (Levene & Gross, 1959); the effect was also observed in the embryos of lathyritic animals (Stamler, 1955), in a tumour implanted into animals subsequently rendered lathyritic (McCrary, Akamatsu & Orbison, 1963) and in organ cultures treated with a lathyrogen (Schryver & Biggers, 1963).

The present study was undertaken to ascertain whether this was a specific effect of all lathyrogenic compounds and whether it was related to the clearly established lesion of collagen produced by BAPN, namely a failure of polymerization at the intra- and inter-molecular level.

The tissue selected was cartilage, partly because its relative avascularity tends to eliminate inflammatory exudate as a cause of increased hydration and partly because many quantitative and qualitative data were available for various purified components isolated from normal and lathyritic cartilage (Levene, Kranzler & Franco-Browder, 1966).

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† Abbreviation: BAPN, β-aminopropionitrile.

The results suggest that the increased hydration is specifically due to the lathyrogenic compound and that the phenomenon is probably closely related to the collagen defect.

EXPERIMENTAL

Materials. Eighteen fertile chick-embryo eggs were incubated under the usual conditions of temperature and humidity, and injected at 14 days of incubation with 5mg. of BAPN (generous gift from Abbott Laboratories, North Chicago, Ill., U.S.A.) dissolved in 0·1ml. of distilled water through a pinhole in the shell on to the chorioallantoic membrane and reincubated. The survivors were harvested 2days later by rapidly dissecting the tibiae and femora clean of muscle and tendon, and pooling the cartilaginous ends, free of bone, in a closed vial to avoid evaporation of water. They were then divided into three portions, carefully weighed, and dried for 2days *in vacuo* at 105° to a constant weight in an Abderhalden drying pistol; the percentage dry weight was calculated from the average of the three samples; experiments containing obvious discrepancies due to possible loss *in vacuo* of fragments of cartilage were discarded and the experiment was repeated.

Testing the lathyrogenic compounds and non-lathyrogenic analogues. This was accomplished as follows. Each complete experiment included a normal uninjected control, a known lathyrogenic compound, and four non-lathyrogenic analogues. The dosage for each test compound was 0·054 m-mole dissolved in 0·1ml. of water/egg (the LD_{50} for BAPN in 14-day-old chick embryos) and 18 14-day-old embryos were used for each compound tested and for the

Table 1. *Effect of BAPN on chick-embryo weight and percentage dry weight of long-bone cartilage*

BAPN (5mg.) was injected at 14 days and the embryos were examined 2 days later. The value for each dry-weight determination was taken as the average of three separate fractions; the results are expressed as means ± S.E.M., with the numbers of determinations in parentheses.

	Embryo wt. (g.)		Dry wt. of cartilage (%)	
Normal controls at 16 days	14·7±0·27 (12)	$P>0.5$	13·8±0·21 (10)	$P<0.001$
BAPN-treated embryos at 16 days	14·7±0·48 (8)		11·8±0·07 (10)	

Table 2. *Effect with time of a single dose of BAPN on the percentage dry weight of long-bone cartilage and bony shaft*

BAPN (5mg.) was injected into 14-day-old chick embryos; each value represents the average of three separate fractions.

Embryo age (days)	Dry wt. of cartilage (%)		Dry wt. of bone (%)	
	Normal	BAPN-treated	Normal	BAPN-treated
14	13·6	—	24·5	—
16	15·0	13·0	30·6	29·6
18	16·2	13·6	31·1	29·0
20	17·8	17·3	33·7	31·3

uninjected normal controls. The harvesting of cartilage 2 days later, at 16 days, and the dry-weight estimations, were performed as described above.

Measurements of relative viscosity. These were made on M-NaCl extracts of the fresh cartilaginous ends of tibiae and femora by the method of Levene & Gross (1959); they are a good index of the amount of collagen present in solution, the relationship being almost linear (Gross, 1958).

RESULTS

Treatment with BAPN produced a significant increase in the hydration of cartilage, though not in the embryo weight (Table 1); calculation from the average percentage dry weight values showed that, whereas 100g. of normal dry cartilage combines in the fresh state with 624g. of water, 100g. of lathyritic dry cartilage combines with 749g. of water, an increase of 20%.

When a single dose of 5mg. of BAPN was injected into 14-day-old chick embryos the hydration effect did not subside until about 6 days later; there was also an increase in the hydration of the separately dissected bony shafts of the tibiae and femora; it was of a smaller magnitude and had not completely subsided by 6 days (Table 2 and Fig. 1).

It was decided to test whether this increase in the water content was real or apparent and whether it was associated with an increase or decrease in the absolute dry mass per long bone. Therefore the wet and dry weights of the complete femur were measured, since separation into cartilage and bony shaft was bound to bring in errors due to faulty anatomical delineation between cartilage and bone.

The results show that there was a slight increase in the absolute amount of water per lathyritic femur, accompanied by a small decrease in the absolute dry mass; the percentage dry weight/bone increased with age (Table 3).

Specificity of lathyrogenic compounds. In the past a lathyrogenic compound has been defined as one that produces an increase in the fragility and salt-solubility of the collagen from the connective tissues of the chick embryo (Gross, Levene & Orloff, 1960). Such compounds may be classified into four groups: (*a*) certain organic nitriles, e.g. BAPN; (*b*) ureides, e.g. semicarbazide; (*c*) hydrazides, e.g. isonicotinic acid hydrazide; (*d*) hydrazines, e.g. hydrazine hydrate (Levene, 1961*b*).

To test whether the change in collagen induced by lathyrogens *in vivo* was always accompanied by an increase in the hydration of cartilage, a number of known lathyrogenic compounds were investigated, together with a number of non-lathyrogenic but closely related compounds. The results indicate that the increase in hydration is specific to lathyrogenic compounds (Tables 4 and 5).

Dose–response relationship. One lathyrogen from each of the four lathyrogenic groups of compounds was tested at different dosages for its effect on both the hydration of cartilage and the amount of collagen rendered soluble in cold M-sodium chloride, as measured by the relative viscosity of the extracts; the embryos were injected at 14 days and examined 2 days later.

The results indicate that for all of these compounds there appears to be, at the lower dosages, a fairly direct relationship between dosage and the percentage of excess of hydration induced in the cartilage, as well as between the increase in hydration and the amount of salt-soluble collagen extractable with M-sodium chloride (Fig. 2).

Effect of pyridoxal or glyceraldehyde. The collagen effect induced in chick embryos by certain lathyro-

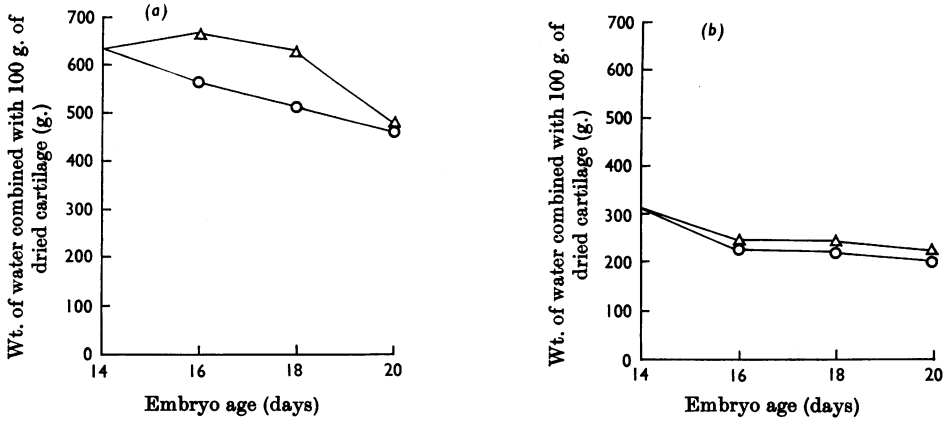

Fig. 1. Effect of a single dose of BAPN (5mg. at 14 days) on the subsequent state of hydration of cartilage (*a*) and bony shaft (*b*) of chick-embryo long bones. Each value represents the average of three separate determinations in one experiment. ○, Normal; △, lathyritic.

Table 3. *Effect of a single dose of BAPN on the subsequent chick-embryo weight and on the fresh and dry weights of the complete femur*

BAPN (5mg.) was injected into 14-day-old chick embryos; each value represents the average of ten pooled specimens in one experiment.

Embryo age (days)	Embryo wt. (g.)		Average wt. of fresh femur (mg.)		Average wt. of dry femur (mg.)		Dry wt. of femur (%)	
	Normal	BAPN-treated	Normal	BAPN-treated	Normal	BAPN-treated	Normal	BAPN-treated
14	9·6	—	28·5	—	5·0	—	17·3	—
16	14·7	14·7	61·4	62·0	12·0	10·6	19·6	17·1
18	20·4	19·7	116·5	119·5	26·9	23·9	22·9	19·9
20	28·7	33·4	153·8	153·6	38·2	34·7	25·0	22·6

gens, particularly by isonicotinic acid hydrazide but not by BAPN, is largely reversible with pyridoxal treatment and, to a smaller extent, with glyceraldehyde (Levene, 1961*a*). It was therefore decided to test whether the increased hydration of cartilage induced in 16-day-old chick embryos by treatment at 14 days with various lathyrogens could be modified by injecting these embryos with pyridoxal or glyceraldehyde at 15 days and examining them at 16 days, together with the appropriate controls.

The results (Table 6) indicate that neither pyridoxal nor glyceraldehyde has any obvious effect on the hydration of cartilage either in the lathyritic embryos or the normal controls.

Effect of an antihistamine. Diphenhydramine (Benadryl; Parke, Davis and Co., Hounslow, Middlesex) inhibits the movement of water in cells damaged by toxic agents (Judah, 1960). This effect was made use of in ascertaining whether the increased hydration of lathyritic cartilage was due primarily to cell damage rather than to extracellular causes. To test this, chick embryos that had been treated at 14 days with 5mg. of BAPN were treated at 15 days with 2·5mg. of Benadryl (the approximate LD_{50} as determined by a preliminary trial), and the survivors harvested at 16 days. The dry weight of long-bone cartilage was determined, together with that of the appropriate controls; the results (Table 7) indicate that Benadryl treatment did not modify the hydration effect of BAPN.

DISCUSSION

In general, slightly more than one-third of cartilage water is intracellular; half of the remaining extracellular water has been assigned to the collagen and 'associated' mucopolysaccharide and the other

Table 4. *Effect of lathyrogenic compounds and non-lathryogenic structurally related analogues on the state of hydration of chick-embryo long-bone cartilage*

Each compound (0·054 m-mole) was injected into 14-day-old chick embryos and the cartilage was examined 2 days later; each value represents the average of three separate fractions in one experiment. Hydration index is defined as:

$$\frac{\text{Water content of 'treated' cartilage}}{\text{Water content of same dry mass of normal cartilage}}$$

Compound (0·054 m-mole/egg at 14 days)	Formula	Lathyrogenic ability	Dry wt. of cartilage of treated embryo at 16 days (%)	Dry wt. of cartilage of normal control embryo at 16 days (%)	Hydration index
Aminoacetonitrile	$H_2N \cdot CH_2 \cdot CN$	+	11·50	15·15	1·37
Glycine	$H_2N \cdot CH_2 \cdot CO_2H$	−	13·70	13·68	1·00
Methylamine	$H_2N \cdot CH_2 \cdot H$	−	13·93	13·68	0·98
Glycine methyl ester	$H_2N \cdot CH_2 \cdot CO \cdot O \cdot CH_3$	−	13·80	14·05	1·02
Aminoacetonitrile	$H_2N \cdot CH_2 \cdot CN$	+	11·50	15·15	1·37
Methyleneaminoacetonitrile	$CH_2{:}N \cdot CH_2 \cdot CN$	+	12·20	14·33	1·20
Cyanoacetic acid	$HO_2C \cdot CH_2 \cdot CN$	−	14·54	15·15	1·05
Acetonitrile	$H \cdot CH_2 \cdot CN$	−	15·03	15·15	1·01
Propionitrile	$CH_3 \cdot CH_2 \cdot CN$	−	14·08	14·05	1·00
β-Aminopropionitrile (BAPN)	$H_2N \cdot CH_2 \cdot CH_2 \cdot CN$	+	11·90	14·00	1·21
β-Mercaptoethylamine	$H_2N \cdot CH_2 \cdot CH_2 \cdot SH$	−	14·03	14·05	1·02
Ethylenediamine	$H_2N \cdot CH_2 \cdot CH_2 \cdot NH_2$	−	14·75	14·05	0·94
β-Aminopropionitrile	$H_2N \cdot CH_2 \cdot CH_2 \cdot CN$	+	11·90	14·00	1·21
β-Hydroxypropionitrile	$HO \cdot CH_2 \cdot CH_2 \cdot CN$	−	13·65	13·68	1·00
Semicarbazide	$H_2N \cdot NH \cdot CO \cdot NH_2$	+	11·20	14·05	1·30
Acetone semicarbazone	$(CH_3)_2C{:}N \cdot NH \cdot CO \cdot NH_2$	+	11·20	14·05	1·30
Acetamide	$CH_3 \cdot CO \cdot NH_2$	−	15·20	15·15	1·00
Urea	$H_2N \cdot CO \cdot NH_2$	−	13·85	14·05	1·02
Nicotinamide	$C_5H_4N \cdot CO \cdot NH_2$	−	13·75	14·05	1·03
1-Phenylsemicarbazide	$C_6H_5 \cdot NH \cdot NH \cdot CO \cdot NH_2$	−	13·88	14·33	1·04
L-Asparagine	$H_2N \cdot CH(CO_2H) \cdot CH_2 \cdot CO \cdot NH_2$	−	14·85	15·15	1·02
L-Glutamine	$HO_2C \cdot CH(NH_2) \cdot [CH_2]_2 \cdot CO \cdot NH_2$	−	13·88	13·68	0·98
Semicarbazide	$H_2N \cdot NH \cdot CO \cdot NH_2$	+	11·20	14·05	1·30
Isonicotinic acid hydrazide	$H_2N \cdot NH \cdot CO \cdot C_5H_4N$	+	11·70	14·05	1·23
Benzohydrazide	$H_2N \cdot NH \cdot CO \cdot C_6H_5$	+	11·75	13·68	1·19
Cyanoacetic acid hydrazide	$H_2N \cdot NH \cdot CO \cdot CH_2 \cdot CN$	+	12·40	14·33	1·18
Thiosemicarbazide	$H_2N \cdot NH \cdot CS \cdot NH_2$	+	11·50	14·05	1·26
4,4-Diphenylsemicarbazide	$H_2N \cdot NH \cdot CO \cdot N(C_6H_5)_2$	−	14·20	13·68	0·96
Hydrazine hydrate	$H_2N \cdot NH_2,H_2O$	+	11·10	14·05	1·31
sym-Dimethylhydrazine	$CH_3 \cdot NH \cdot NH \cdot CH_3$	+	12·55	14·33	1·17

half to the free interstitial water (Eichelberger, 1960; Miles & Eichelberger, 1964). The failure of an antihistamine to prevent the increased hydration in lathyritic cartilage suggests that the excess of water is extra- rather than intra-cellular. The quantity and distribution of extracellular water in cartilage depends on the ionic state and on the state of the collagen, acid mucopolysaccharides, non-collagenous proteins and macromolecular aggregates, as well as on the excluded volume effect; however, very little is known about these.

The fixed ionic groups on chondroitin sulphate, a polyanion, play a major role in water imbibition (Kantor & Schubert, 1957); the diffusible ions, e.g. chloride, help to regulate water movement in cartilage (Linn & Sokoloff, 1965).

Collagen is regarded as a special case in relation to water because, unlike silk fibroin, DNA and keratin, it possesses many hydrogen-bond sites for water, which forms structural chains in the fibre direction (Berendsen & Migchelsen, 1965); ions such as phosphate are believed to exert their stabilizing effect on

the native structure of collagen by acting directly on this structural water (Von Hippel & Wong, 1964; Berendsen & Migchelsen, 1965).

The role of the acid mucopolysaccharides in the hydration of cartilage is obscure; the hyaluronic acid–water relationship has been well studied, particularly the excluded volume effect (Ogston & Phelps, 1961), but chick-embryo cartilage contains only chondroitin sulphates A and C. The suggestion that these may behave like hyaluronic acid (Fessler, 1960) appears to be supported by the available but limited evidence (Gerber & Schubert, 1964; Milch, 1965). The state of aggregation of macromolecules in cartilage is also an important factor in determining its water and ionic content (Gersh & Catchpole, 1959–60).

In spite of their importance no data are available for lathyritic cartilage on ionic conditions, non-collagenous proteins, states of macromolecular aggregation or changes in excluded volume of acid mucopolysaccharides. It is, however, established that lathyritic collagen possesses a cross-linking defect, whereas chondroitin sulphate and its protein complex from the same cartilage are normal, when examined qualitatively and quantitatively, chemically and physicochemically (Levene *et al.* 1966). It is therefore possible only to present the available evidence that the lathyritic collagen molecule is

Table 5. *Effect of lathyrogenic compounds on the state of hydration of the long-bone cartilage of the chick embryo compared with the effect of structurally related but non-lathyrogenic analogues*

Each compound (0·054 m-mole) was injected into 14-day-old chick embryos and the cartilage was examined 2 days later. Each value represents the average of three separate fractions; the results are expressed as means ± S.E.M., with the numbers of determinations in parentheses. *P* values were calculated for differences between groups 1 and 2 (> 0·90), groups 1 and 3 (< 0·01) and groups 2 and 3 (< 0·01).

Group	Dry wt. of cartilage (%)
1. Normal untreated controls	14·2 ± 0·21 (6)
2. Non-lathyrogenic controls	14·2 ± 0·49 (16)
3. Lathyrogenic embryos	11·7 ± 0·03 (10)

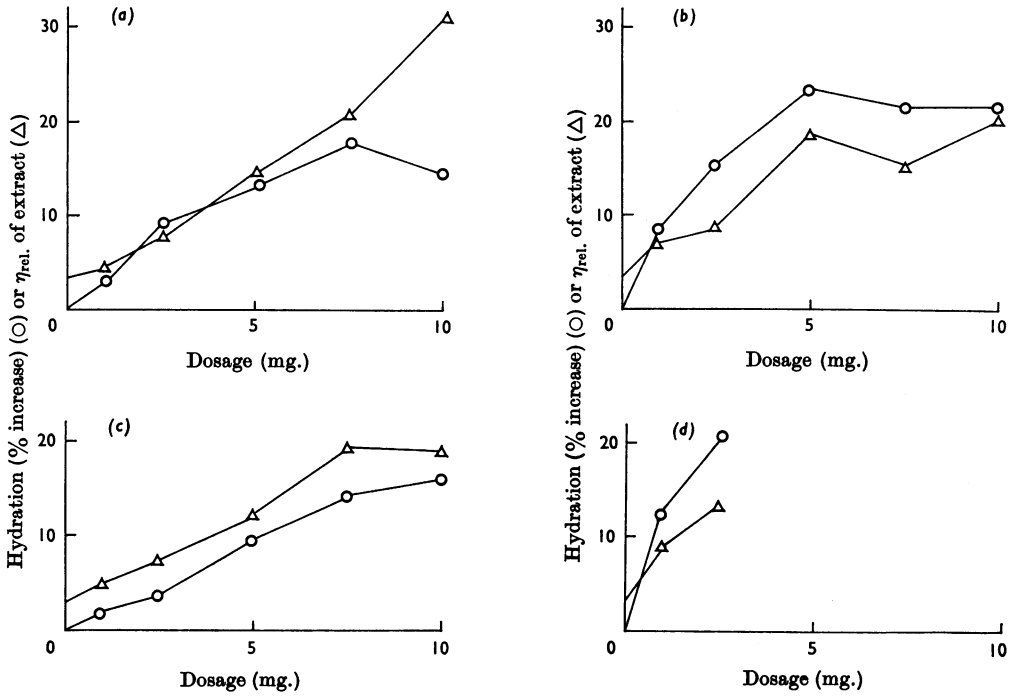

Fig. 2. Correlation, at various lathyrogen dosages, between the excess of hydration and the increase in collagen solubility produced in the long-bone cartilage of 16-day-old chick embryos injected 2 days earlier. ○, Hydration value as percentage increase over normal, each value representing the average of three separate determinations in one experiment; △, relative viscosity of M-NaCl extracts of cartilage. (*a*) BAPN; (*b*) semicarbazide; (*c*) isonicotinic acid hydrazide; (*d*) hydrazine hydrate.

Table 6. *Effect of pyridoxal or glyceraldehyde on the hydration effect of lathyrogenic compounds on chick-embryo long-bone cartilage*

The lathyrogenic compound (0·054m-mole) was injected into 14-day-old chick embryos; 5mg. of pyridoxal or glyceraldehyde was injected 1 day later and the cartilage examined 1 day after this. Each value represents the average of three separate fractions in one experiment.

		Dry wt. of cartilage (%)						
	Embryo treatment	β-Amino-propio-nitrile (BAPN)	Semi-carbazide	Isonico-tinic acid hydrazide	Hydrazine hydrate	Amino-aceto-nitrile	Thiosemi-carbazide	Cyano-acetic acid hydrazide
Controls	Normal	13·5	13·1	13·6	12·6	13·0	13·8	13·2
	Lathyrogenic	11·5	10·8	11·1	10·9	11·2	11·5	11·9
	Pyridoxal	13·3	13·1	13·2	—	—	—	—
	Glyceraldehyde	13·7	13·2	13·7	—	—	—	—
Lathyrogen + pyridoxal		11·8	11·6	11·8	10·9	11·2	10·9	11·9
Lathyrogen + glyceraldehyde		11·7	11·5	11·4	11·3	10·9	11·3	11·9

Table 7. *Effect of Benadryl treatment on the hydration effect of BAPN on chick-embryo long-bone cartilage*

Each value represents the average of three separate samples.

		Dry wt. of cartilage (%)
Controls	Normal	14·0
	Benadryl (2·5mg. at 15 days)	13·6
	BAPN (5·0mg. at 14 days)	12·0
BAPN (5mg. at 14 days) + Benadryl (2·5mg. at 15 days)		11·6

abnormal, and probably more hydrated than usual, based on chain sub-unit studies and X-ray diffraction.

The work of Martin, Gross, Piez & Lewis (1961), confirmed by Nikkari & Kulonen (1962), indicates an intramolecular defect in cross-linking of the component chains in the lathyritic tropocollagen molecule. An X-ray diffraction study (Kundel, 1964) showed that, whereas normal and lathyritic rat-tail tendon had identical wide-angle patterns in the dry state, hydration produced a suggested increase in the equatorial spacing of the lathyritic collagen over the normal; Kundel (1964) interpreted this as meaning that the blockage of intramolecular bonds by lathyrogens allowed a wider separation of the chains in the collagen molecule than normal. The evidence given in the present paper strongly suggests that the amount of excess of hydration in lathyritic cartilage is paralleled by the amount of salt-soluble collagen produced, and that these are both dependent, within limits, on the dosage of lathyrogen. This tends to implicate the physical state of the collagen molecule as a cause of the increased hydration. However, the increased hydration might be due to the increased imbibition of water by chondroitin sulphate–protein complexes when released from the normal restraint of the tightly bound collagen network.

This work was made possible through the help of U.S. Public Health Grant no. AM-05996. The author acknowledges the facilities granted him by Dr A. Dorfman, the efforts of Miss Judith Kaufman, the skilled and patient assistance of Miss Jean Hayashi and helpful discussion with Dr E. Kodicek.

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