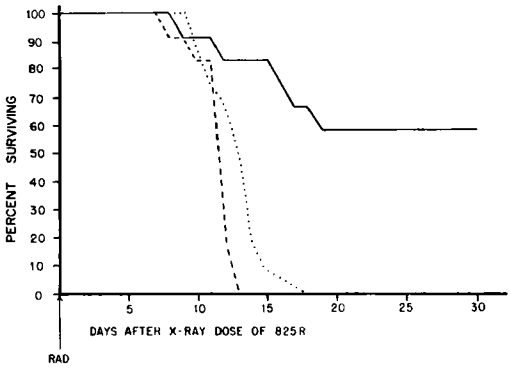

Fig. 2—Radioprotection by 7,10-ethano-1-thia-4,7-diazaspiro [4.5] decane dihydrochloride injected i.p. in mice 15 min. before X-radiation. Key: —, 0.2 mg./g. drug; · · ·, control; - - -, 0.02 mg./g. drug.

sure of mice to 825 r. Since the absorption and fate of the test compound has not yet been established, it is not known whether the failure to protect was due to the route of administration or the lack of absorption of the compound in the form of the dihydrochloride salt.

The mechanism(s) by which 7,10-ethano-1-thia-4,7-diazaspiro [4.5] decane dihydrochloride enhances the radiation resistance of rodents is not clear. However, the fact that the agent provides protection when it is administered intraperitoneally 15 min. before radiation exposure, places it in the same category as the classical chemical radioprotectors such as cysteine, cysteamine, or AET which are only effective when administered immediately before radiation.

The synthesis and radioprotective action of several analogs of 7,10-ethano-1-thia-4,7-diazaspiro[4.5]-decane dihydrochloride will be reported in a later publication.

REFERENCES

(1) Löffler, K., and Stiezel, F., *Ber.* **42**, 124(1909).
(2) Sternbach, L. H., and Kaiser, S., *J. Am. Chem. Soc.*, **74**, 2219(1952).
(3) Mikhlina, E. E., and Rubstov, M. V., *Zh. Obshch. Khim.*, **30**, 163(1960); through *Chem. Abstr.*, **54**, 22632 (1960).
(4) Grob, C. A., U. S. pat. 2,917,515 (Dec. 15, 1959).
(5) Rubstov, M. V., Mikhlina, E. E., and Yakhontov, L. N., *Russ. Chem. Rev.* **29**, 44(1960).
(6) Bacq, Z. M., and Herve, A., *Bull. Acad. Roy. Med. Belg.*, **17**, 13(1952).
(7) Pihl, A., and Eldjarn, L., *Pharmacol. Rev.*, **10**, 453 (1958).
(8) Kaluszyner, A., Czerniak, P., and Bergmann, E. D., *Radiation Res.*, **14**, 23(1961).
(9) Bellamy, L. J., "The Infra-red Spectra of Complex Molecules," 2nd ed., Wiley, New York, N. Y., 1958, p. 132.
(10) Grob, C. A., and Zergenyi, J., *Helv. Chim. Acta*, **46**, 2658(1963).
(11) Litchfield, J. T., Jr., and Wilcoxon, F., *J. Pharmacol. Exptl. Therap.*, **96**, 99(1949).
(12) *Ibid.*, **96**, 107(1949).

Keyphrases

Radioprotective agent—quinuclidine derivative
7,10-Ethano-1-thia-4,7-diazaspiro[4.5]decane dihydrochloride—synthesis
IR spectrophotometry—structure
Antiradiation activity—quinuclidine derivative

Preliminary Pharmacology of Ellagic Acid from *Juglans nigra* (Black Walnut)

By U. C. BHARGAVA, B. A. WESTFALL, and D. J. SIEHR*

A crystalline compound was isolated from *Juglans nigra* which by comparison with the known compound was found to be ellagic acid. The ellagic acid injected (i.p.) produced significant sedation, ataxia, potentiated sodium pentobarbital sleeping time, and protected mice from death after electroconvulsive shock. Intravenous injection of ellagic acid caused a fall in blood pressure and an elevation of the T wave, whereas the heart and respiration rate initially increased followed by a decrease. The ellagic acid produced no significant effect on isolated duodenal and uterus segments of the rat.

DURING THE isolation of sedative principles from *Juglans nigra*, a crystalline compound was obtained and characterized as ellagic acid by comparing it with the known compound. Ellagic acid is a polyphenolic constituent of many plants such as the pericarp tissue of some castor varieties, cashew nut shell, and some walnut species. *Juglans nigra* is a naturally grown plant in eastern and central North America.

Westfall *et al.* (1) observed that the ether ex-

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tract from hulls of *Juglans nigra* has a depressant property in different species of animals. Perhaps the use of walnuts as a vermifuge in the past also might be related to the immobilizing effect of the extracts on worms (2). Fiedler and Hildebrand (3) studied the pharmacology of ellagic acid to some extent and showed that infusion of ellagic acid caused a fall in blood pressure and an increase in respiration in the guinea pig. Similar results were found in the dog by Botti and Ratnoff (4). Blumenberg *et al.* (5) fed rats with ellagic acid (50 mg./kg., orally) up to a maximum of 45 days. Histological examination of liver and other tests showed no evidence of toxicity in animals.

In this present study, some additional pharmacological properties of ellagic acid were established.

EXPERIMENTAL

Isolation and Properties of Ellagic Acid—Two kilograms of hulls of *Juglans nigra* were extracted (6) with diethyl ether–butanol mixture (4:1). The extract was evaporated under vacuum and triturated with 5% acetic acid. The acetic acid solution was extracted several times with diethyl ether–butanol mixture. The organic solvent phase was collected and washed several times with $NaHCO_3$ solution at pH 7.5 and the alkaline solution was acidified and extracted with diethyl ether–butanol mixture. During the evaporation of the organic solvent phase under vacuum, a small quantity of dark brown powder was obtained. It was washed with cold ethanol, dried, and was crystallized from a large volume of acetone as pale yellow crystals (10 mg. crystals per kg. of hulls). In succeeding experiments, it was found that the yield of the yellow crystalline material could be increased (35 mg. crystals per kg. of hulls) with reproducible results, if the residue was triturated with 40% glacial acetic acid and the solvent, after filtration, evaporated in a flash evaporator. Most of the crude crystals separated during the evaporation. Additional crystalline material was recovered on cooling. The crude material was recrystallized from pyridine.

The high melting point[1] (dec. >360°), the blue coloration with ferric chloride, a positive Greissmeyer reaction (7), its behavior on a paper chromatogram (8), and the IR and UV absorption spectra of the crystalline compound isolated from black walnut hulls were properties identical to those of an authentic sample[2] of ellagic acid. IR and UV absorption spectra were compared using a Beckman IR 7, and a Cary model 14 spectrophotometer, respectively.

Anal.[3]—Calcd. for $C_{14}H_6O_8 \cdot 2H_2O$: C, 49.7; H, 2.96. Found: C, 48.61; H, 3.21.

This material was almost insoluble in the usual organic solvents and water, but dissolved in hot pyridine and produced a yellow solution in aqueous NaOH. For injection walnut ellagic acid was dissolved in 0.1*N* NaOH solution and the pH adjusted to 10 for most of the preparations. A high pH was essential to keep ellagic acid in solution.

Animals—Swiss Webster male mice (30–40 g.) and Wistar male (220–280 g.) and nonpregnant female rats (170–220 g.) were used. Mice were housed in groups of five and rats in four. On their arrival from the farm, they were fed Purina chow and water *ad libitum*. At least a week was allowed after the shipment before the animals were used. The rats were anesthetized with sodium pentobarbital (50 mg./kg. i.p.) for blood pressure measurement, and were killed by concussion for isolated tissue preparations.

Spontaneous Activity—A "jiggle" type activity cage was used for measuring activity. The vibrations originated by the movements of the mouse in the cage were picked up by a phonograph-type crystal pick-up cartridge and converted into electrical impulses which were recorded automatically by a counter. Ninety minutes after ellagic acid administration (1–20 mg./kg. i.p.) a mouse was placed in each activity cage and after 30 sec. activity counts were measured for 5 min.

Ataxia—For measuring muscular incoordination, a rotating rod of diameter 3.5 cm., with a speed of 5 r.p.m. and located 10 in. above the table was used (9). Immediately after measuring the spontaneous activity, the mouse was transferred to the rotating rod and the time required for falling off (before 5 min.) for each mouse was recorded. Two opportunities were given to each mouse on the rotating rod, and if the given mouse remained for more than 5 min. in either of the two attempts, "yes" was recorded, otherwise "no." From these observations the proportion of mice remaining on the rotating rod was calculated.

Sodium Pentobarbital Sleeping Time—Ninety milligrams of ellagic acid/kg. of body weight was injected (i.p.) 90 min. prior to sodium pentobarbital administration. Then a dose of 50 mg./kg. of sodium pentobarbital was injected (i.p.) for narcosis. After the mice lost the righting reflex, they were left on their backs and their tails were pinched 1 cm. distal to the anus every 5 min. Pressure of pinching the tails was kept approximately the same every time. The sodium pentobarbital sleeping time was the time from loss of the righting reflex to return of the righting reflex.

Anticonvulsive Activity—At the peak response period which was 90 min. after the ellagic acid administration (180 mg./kg. i.p.), electroconvulsive shock of 24 ma. for 0.2 sec. (a.c.) was applied through ear clips (with electrode paste) to each mouse (10). The abolition of seizure (tonic extensor) was indicative of anticonvulsant effect of drug. Usually after such electroshock, the untreated mouse dies. Therefore, the number of animals protected by ellagic acid from this type of death was calculated.

Blood Pressure—Blood pressure in rats was measured through the femoral artery using a Statham pressure transducer. Respiration was recorded by inserting one end of tubing into the trachea and connecting the other end to a volumetric pressure transducer (PT-5-A). Lead II was used for recording the ECG. All responses were recorded

[1] Melting point was determined by the Galbraith Laboratories, Inc., Knoxville, Tenn., using Arthur Thomas apparatus.

[2] An authentic specimen of ellagic acid was purchased from Aldrich Chemical Co., Milwaukee, Wisc.

[3] Microanalysis of ellagic acid was also conducted by the Galbraith Laboratories.

by polygraph (Grass). Heart rate was calculated from the ECG. Five milligrams of ellagic acid/kg. was injected into the femoral vein and not more than 0.5 ml. solution was injected at one time.

Rat Duodenum—Two duodenal segments, approximately 3 cm. long, were taken about 1 cm. distal from the pyloric region of the stomach. These were suspended in Tyrode's solution and maintained at a temperature of 37°. The solution was aerated by means of a steady stream of O_2. One preparation was used as control and another as experimental. Recordings of duodenal activity of five animals were obtained by means of a force displacement transducer attached to polygraph (Grass). After obtaining normal responses, ellagic acid (0.01 mg./ml.) was added to one of the preparations and the other was considered as a control.

Rat Uterus—A uterine segment 3 cm. in length was removed from each uterine horn of 5 animals and they were treated in similar manner as the duodenal segments except 0.05 mg./ml. ellagic solution was added instead of 0.01 mg./ml.

RESULTS AND DISCUSSION

A crystalline product was isolated from the hulls of *Juglans nigra* whose elemental analysis, melting point, UV, and IR spectra were identical to those of synthetic ellagic acid as shown in Figs. 1 and 2. The crystalline material could not be separated from synthetic ellagic acid when the substances were cochromatographed on paper.

Injection of ellagic acid in amounts from 1 mg./kg. to 20 mg./kg. caused a progressive increase in sedation as shown in Fig. 3. When the animals were placed on the rotating rod, there was a significant decrease in the proportion of mice (which received ellagic acid) remaining on the rotating rod as illustrated in Fig. 4. Ellagic acid injected (90 mg./kg. i.p.) 90 min. prior to sodium pentobarbital

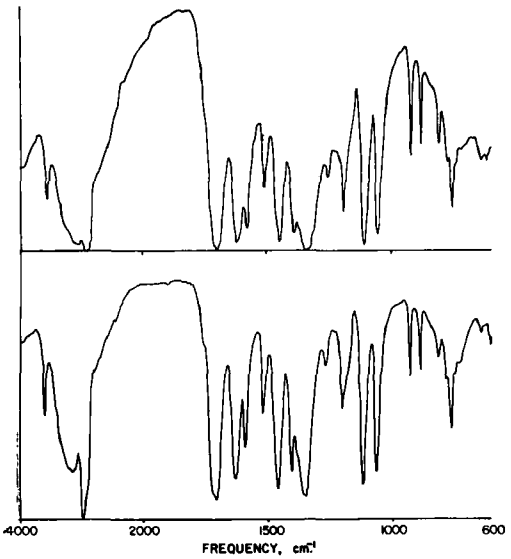

Fig. 1—IR spectra of natural and synthetic ellagic acid in mineral oil. Key: top, synthetic ellagic acid; bottom, ellagic acid from black walnut.

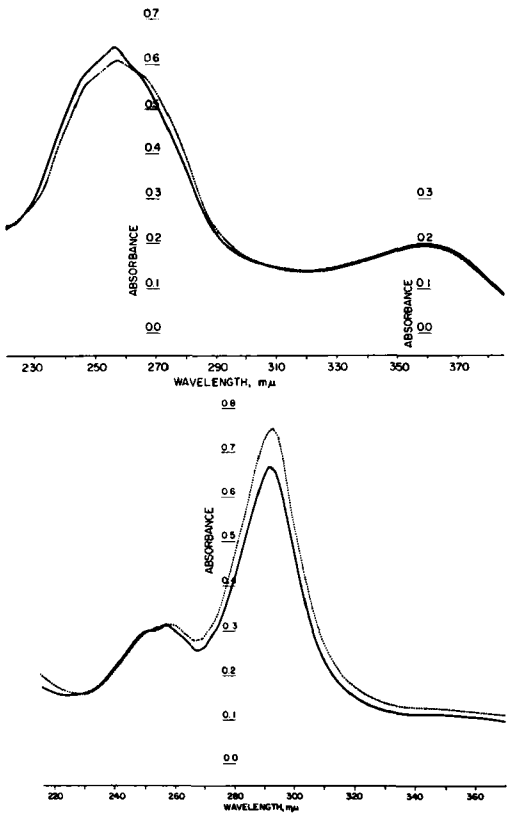

Fig. 2—UV absorption spectra of natural and synthetic ellagic acid in absolute alcohol and NaOH solution. Key: Top (absolute alcohol); bottom (in NaOH solution); . . ., synthetic ellagic acid; —, ellagic acid.

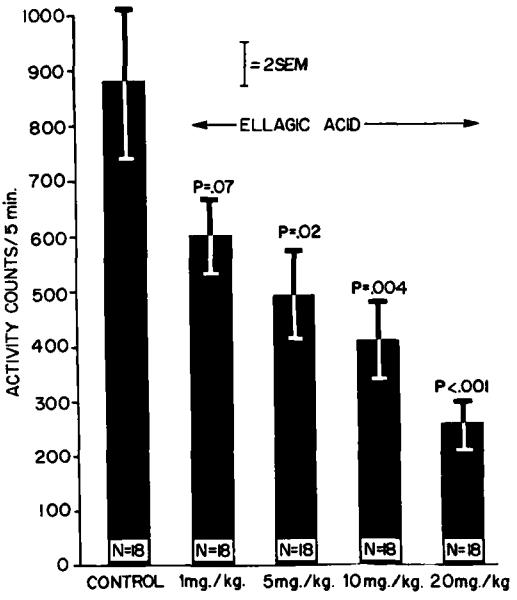

Fig. 3—Effect of ellagic acid on spontaneous activity in mice. Each bar was obtained from the mean ± S.E.M. of the 18 animals. Statistical significance (p value) was calculated by comparing the experimental group with the control.

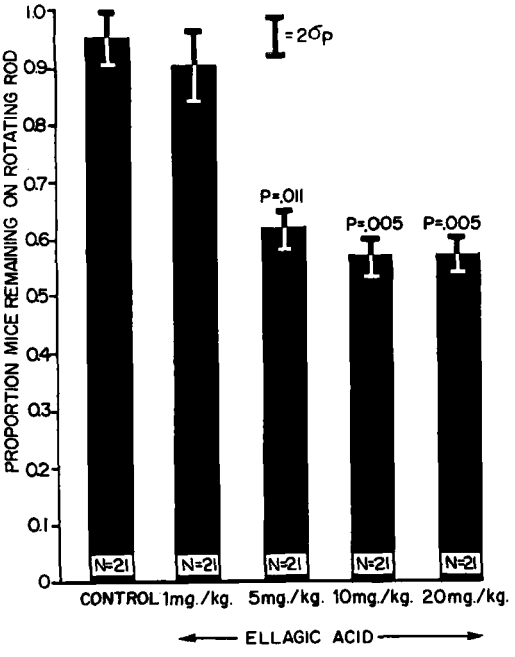

Fig. 4—Effect of ellagic acid on muscular incoordination in mice. Each bar was obtained from the proportion ± S.E.P. of the 21 animals which received "yes." Statistical significance (p *value) was calculated by comparing the experimental group with the control.*

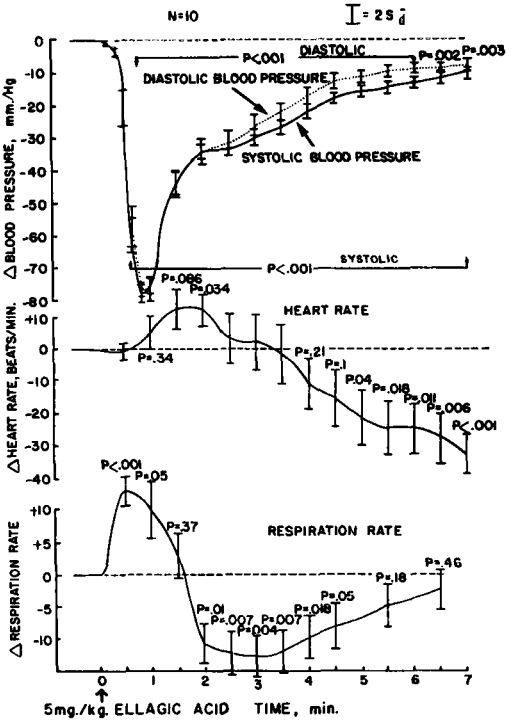

Fig. 5—Effect of ellagic acid on blood pressure, heart rate, and respiration rate in rats. Each point was obtained from the mean ± SE of the 10 animals. Statistical significance (p *value) was calculated by testing the differences.*

injection appeared to potentiate significantly ($p < 0.001$) the sleeping time (control: 34.5 ± 3.6 min. S.E.M.; experimental: 64.5 ± 4.2 min. S.E.M.), but without sodium pentobarbital, the mice receiving ellagic acid did not sleep at all. Ellagic acid (180 mg./kg. i.p.) did not show any anticonvulsive activity (χ^2 test). However, the number of mice remaining alive after electroconvulsive shock was increased. Seven out of 17 animals remained alive in the controls as compared to 14 out of 17 animals in the experimental group. These types of action seem to be more like tranquilizers than sedatives. Furthermore, as there are very few drugs which protect from electroshock, ellagic acid might be potentially useful in this area.

As illustrated in Fig. 5, intravenous injection of ellagic acid (5 mg./kg.) in rats caused a fall in blood pressure, whereas an equal volume of saline at pH 10 produced no change in pressure. In most of the animals there was a lag period of approximately 20 sec. before blood pressure began to fall and the maximum drop in blood pressure was approximately 1 min. after drug administration. Five minutes later blood pressure approached a steady level which was somewhat less than a control level. Heart rate first increased and then decreased and the decrease was more pronounced. Respiration rate first increased then decreased and after 5 min. no significant difference from the control was observed. During the blood pressure fall, the amplitude of respiration was also increased. In the ECG, the T wave was elevated approximately after 2 min. of ellagic acid administration. Similar results were obtained by other workers using guinea pig and dog (3, 4).

There was no apparent effect observed when ellagic acid in a concentration of 0.01 mg./ml. was added in the tissue bath of duodenal segments. There was a slight increase in amplitude and rate of contractions of rat uterine strips in some of the animal preparations used, but there was inconsistency in these results. Therefore, not much weight could be put on this experiment unless a large number of animals were studied and the animals divided according to estrus and nonestrus cycle, since the sexual cycle can play an important part in response to uterine tone and frequency of contractions (11).

Pharmacological tests of ellagic acid on a broader scale are currently in progress. In order to evaluate other active principles present in *Juglans nigra*, crude fractions (6) such as strong acids and weak acids were also injected into rats (i.v.). Like ellagic acid both strong acids and weak acids (20 mg./kg. i.v.) produced a fall in blood pressure, elevation of the T wave, and respiration rate initially increased, then decreased. However, after injection of strong acids the onset of the blood pressure fall was much slower and the maximum drop observed occurred between 3 and 3.5 min. after administration of strong acids, whereas after injection of weak acids the return of blood pressure toward normal was much slower.

SUMMARY

1. A crystalline compound isolated from black walnut hulls was found to be ellagic acid.

2. Intraperitoneal injection of ellagic acid in mice produced significant sedation, ataxia, potentiation of sodium pentobarbital sleeping time, and protected the animals from death after electroconvulsive shock.

3. Intravenous injection of ellagic acid decreased blood pressure and elevated the T wave. Whereas heart and respiration rate initially increased then decreased.

4. *In vitro* studies indicated that ellagic acid appeared to have no apparent effect on activity of the duodenal segment and uterus of the rat.

REFERENCES

(1) Westfall, B. A., Russell, R. L., and Auyong, T. K., *Science*, **134**, 1617 (1961).
(2) Wood, G. B., and Bache, F., "Dispensatory of the United States," Grigg Elliott, Philadelphia, Pa., 1849.
(3) Fiedler, V., and Hildebrand, G. H., *Arzneimittel-Forsch.*, **4**, 426(1954).
(4) Botti, R. E., and Ratnoff, O. D., *J. Lab. Clin. Med.*, **64**(3), 385(1964).
(5) Blumenberg, F. W., Enneker, C., and Kessler, F. J., *Arzneimittel-Forsch.*, **10**, 233(1960).
(6) Bhargava, U. C., Ph.D. Dissertation, University of Missouri, Columbia, Mo., 1967, p. 64.
(7) Jurd, L., *J. Am. Chem. Soc.*, **78**, 3445(1956).
(8) Hathway, D. E., "Chromatographic and Electrophoretic Techniques," vol. 1, Smith, I., Ed., Interscience, New York, N. Y., 1960.
(9) Dunhum, N. W., and Miya, T. S., *J. Am. Pharm. Assoc., Sci. Ed.*, **46**, 208(1957).
(10) Chen, G., Bohner, B., and Ensor, C. R., *Soc. Exptl. Biol. Med.*, **87**, 334(1954).
(11) Handovsky, H., and Daels, J., *G. Rend. Soc. Biol.* **131**, 150(1939).

Keyphrases

Ellagic acid—*Juglans nigra*
Pharmacologic screening—ellagic acid
IR spectrophotometry—identity
UV spectrophotometry—identity

Influence of Dimethyl Sulfoxide (DMSO) on the Percutaneous Absorption of Salicylic Acid and Sodium Salicylate from Ointments

By JOSEPH M. STELZER, JR.*, JOHN L. COLAIZZI, and PAUL J. WURDACK

Dimethyl sulfoxide (DMSO), 15 percent by weight, was incorporated into selected ointment bases containing 10 percent salicylic acid or 11.6 percent sodium salicylate. Percutaneous absorption was studied by determining salicylate blood levels in New Zealand white rabbits at regular intervals for 8 hr. following application of the ointment to the shaved intact skin and confinement by a specially designed bandage. DMSO in hydrophilic ointment and hydrophilic petrolatum produced more rapid drug absorption and higher salicylate blood levels than the control systems. Polyethylene glycol ointment and a polyoxyethylene (20) stearyl ether gel with DMSO did not produce any significant change in the absorption pattern. The salicylate blood levels obtained from percutaneous absorption of sodium salicylate in hydrophilic ointment containing DMSO were lower than with control systems. In the case of hydrophilic petrolatum, there were no significant differences in absorption patterns of sodium salicylate with or without DMSO. Sodium salicylate did not appear to be absorbed from polyethylene glycol ointment whether or not DMSO was included.

AMONG FACTORS which influence percutaneous absorption, Barr (1) included such factors as the condition of the skin, the thermodynamic properties of the medicament, the effects of moisture, the effects of surfactants, the effects of pH, and the effects of the vehicle. He indicated that organic solvents such as ether, chloroform, benzene, and acetone penetrate the skin with ease and enhance the percutaneous absorption of a drug.

The object of the following research was to determine whether dimethyl sulfoxide (DMSO), a relatively nontoxic organic solvent, would alter the percutaneous absorption patterns of salicylic acid and sodium salicylate when incorporated

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