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Step of Dichlorvos Inhibition in the Pathway of Aflatoxin Biosynthesis

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Dichlorvos (dimethyl 2,2-dichlorovinyl phosphate) inhibits the biosynthesis of aflatoxin by *Aspergillus parasiticus*. Cultures treated with dichlorvos excrete an orange pigment which can be converted into aflatoxin B_1 by the untreated mycelia. The orange pigment was partially identified as an acetyl derivative of versiconol-type compound. In the presence of dichlorvos, sterigmatocystin is converted into aflatoxin B_1 without being interfered, but averufin is converted into the orange pigment instead of aflatoxin B_1. Therefore, dichlorvos appears to block an enzymatic step in the aflatoxin biosynthetic pathway, which lies beyond averufin but before sterigmatocystin, at the formation of the orange pigment.

Dichlorvos (dimethyl 2,2-dichlorovinyl phosphate), an organophosphate insecticide, was recently found to inhibit aflatoxin (AF) biosynthesis both in synthetic media (8) and in rice, corn, wheat, and peanut samples inoculated with AF producers (14). Hsieh (8) reported that dichlorvos at 10 ppm inhibited over 90% of AF biosynthesis by *Aspergillus parasiticus* ATCC 15517 grown on different liquid media. Complete inhibition of toxin production occurred with 20 ppm dichlorvos. The treated cultures, however, excreted relatively large quantities of an orange pigment (OP) which is possibly the same pigment recently isolated by Schroeder et al. (17) from cultures of other AF-producing strains treated with dichlorvos. The pigment was probably an acetyl derivative of versiconol-type compound (D. P. H. Hsieh, R. C. Yao, and C. A. Reece, Abstr. Pacific Conf. on Chemistry and Spectroscopy, 1973).

Previously our incorporation studies (9, 12) indicated that sterigmatocystin (ST) and averufin (AR) can be converted into AF B_1 by *A. parasiticus* and are probably intermediates in AF biosynthesis. We also found that AR can be converted into ST by a ST-producing strain of *A. versicolor*. Therefore, experimental support is provided for the biosynthetic scheme for AF B_1 (III) involving AR (I) and ST (II) as proposed by Thomas (18) (Fig. 1). Of particular interest in the scheme is the mechanism of derivation of the bisfuran ring system in the ST and AF molecules from the C_6 side chain in the AR molecule. The biogenetic origin of this unusual structure has been a subject of controversy in the study of *Aspergillus* bisfuranoid biogenesis (2, 6, 7, 13, 18).

In the present study, we show that the aforementioned OP can be converted into AF B_1 by *A. parasiticus* and hence is probably an intermediate in AF biosynthesis, and that dichlorvos inhibits an enzymatic step in the biosynthetic pathway between AR and ST, a step involved in the development of the bisfuran ring system in the molecules of ST and AF B_1 from the C_6 side chain of AR.

MATERIALS AND METHODS

Organisms. The fungi *A. parasiticus* ATCC 15517 and its nitrosoguanidine-derived, averufin-producing mutant, *A. parasiticus* ATCC 24551 (4), and *A. versicolor* ATCC 18643, a sterigmatocystin producer, were used in this study. Conidia were harvested from well-sporulated cultures on mycological agar (Difco) and were stored in 0.01% aqueous sodium lauryl sulfate solution.

Chemicals and media. Technical-grade dichlorvos (Shell Chemical Co., N.Y.), dissolved in acetone, was used in the inhibition studies. Two chemically defined liquid media were used to cultivate the organisms in submerged culture in shaken flasks or in surface culture under static condition. The minimum mineral (MM) medium of Adye and Mateles (1) containing ammonium and 50 g of glucose per liter was used for the production of AF, AR, and OP. Sterigmatocystin was produced by *A. versicolor* in the low-salt (LS) medium of Reddy et al. (16) containing asparagine. The nitrogen-free resting cell (RC) medium of Hsieh and Mateles (11) containing various amounts of glucose was used for incorporation studies and accumulation of OP. All the chemicals used were of reagent grade, and solvents were doubly distilled.

Cultural conditions. Wild-type *A. parasiticus* was

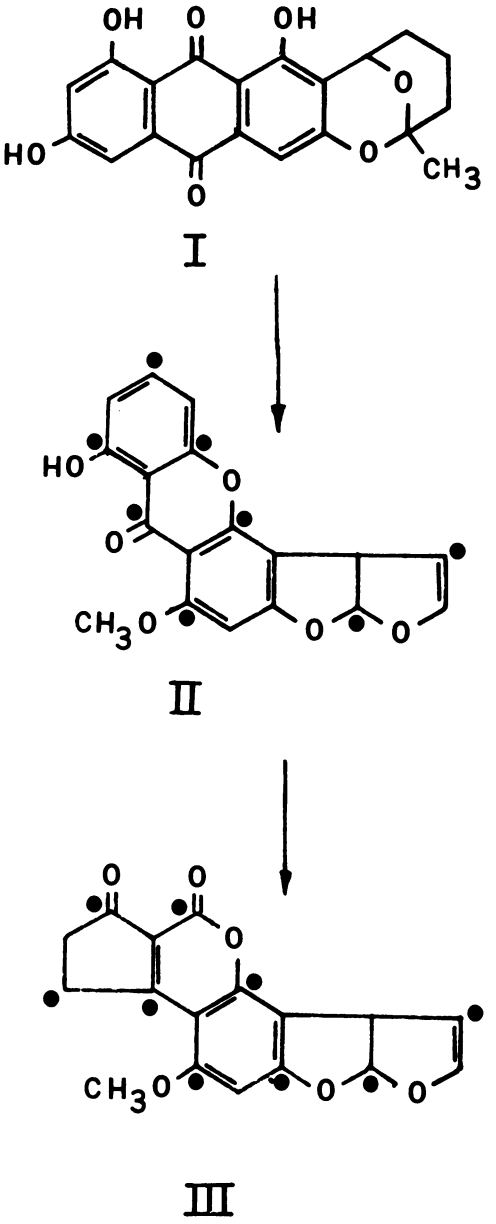

FIG. 1. *Biosynthetic pathway from averufin (I) to sterigmatocystin (II) and aflatoxin B_1 (III) (15). Symbol: ●, labeling pattern derived from [1-^{14}C]acetate (2, 7).*

grown in 100 ml of MM medium in 500-ml indented flasks inoculated with 10^7 conidia. Incubation was done in a gyratory water bath shaker at 30 C, shaking at 100 rpm for the first day and 200 rpm thereafter. The effects of dichlorvos on AF biosynthesis was brought about by incubating 1.5 g of 48-h-old mycelial pellets in 50-ml flasks containing 10 ml of MM or RC medium, to which 0.1 ml of acetone solution containing appropriate amounts of dichlorvos was added.

[^{14}C]averufin was produced as follows. The mutant *A. parasiticus* was grown in 100 ml of MM medium under shake-culture conditions for 24 h. The mycelial pellets were then collected and resuspended in 100 ml of LS medium. Forty-eight hours after transfer, when AR was being accumulated in the mycelia, 0.85 mmol of sodium [1-^{14}C]acetate (0.33 mCi/mmol, New England Nuclear, Boston, Mass.) was added at 24-h intervals for four consecutive days (1 mCi total). The mycelial pellets were collected at the end of the 6th day for extraction and analysis.

[^{14}C]sterigmatocystin was prepared by growing *A. versicolor* in 100 ml of LS medium in a Fernbach flask at 30 C without shaking. After 4 days of growth, 0.4 mmol of sodium [1-^{14}C]acetate (1 mCi/mmol) was added at 24-h intervals for the following 5 consecutive days (2 mCi total). The mycelia were harvested at the end of the 11th day, and ST was extracted with acetone.

^{14}C-labeled OP was prepared by adding [1-^{14}C]acetate to the resting cell culture of wild-type *A. parasiticus* (11), supplemented with 10 ppm of dichlorvos and 36 g of glucose per liter. The pigment was extracted from mycelia after incubation with acetone.

Incorporation of ^{14}C-labeled acetate, AR, ST, or OP into AF B_1 was carried out in RC medium. A 10-ml volume of RC medium containing 300 μmol of glucose was slowly added to 0.1 ml of the methanol solution of each radioactively labeled metabolite contained in a 50-ml indented flask. To each flask was then added 1.5 g of 48-h-old mycelial pellets and an appropriate amount of dichlorvos in acetone. The same amounts of acetone were added to untreated cultures as controls. The flasks were then shaken at 150 rpm at 30 C for 20 h.

Purification and analysis of metabolites. Aflatoxins in the cells and spent medium were exhaustively extracted with acetone and chloroform, respectively. The acetone and chloroform extracts of each flask were pooled and then evaporated to dryness under reduced pressure at room temperature. The residues were taken up in 1 ml of benzene:acetonitrile (98:2, vol/vol). Aflatoxins were purified with a series of thin-layer chromatograms using Adsorbosil-1 (Applied Science Lab, State College, Pa.) plates developed in chloroform:acetone:*n*-hexane (85:15:20), ethylacetate:isopropanol:water (10:2:1), and chloroform:methanol (95:5). The R_f values of AF in the thin-layer chromatography systems were 0.52, 0.71, and 0.62, respectively. Aflatoxin B_1 was identified by co-chromatography with standard and ultraviolet absorption. Quantities of AF were measured with a Schoeffel Spectrodensitometer model SD 3000 (Schoeffel Instrument Corp., Westwood, N.J.).

The acetone extract containing [^{14}C]AR was concentrated under reduced pressure and purified on ChromAR Sheet 500 (Mallinkrodt Chemical Works, St. Louis, Mo.) developed with benzene:acetone (95:5) in unequilibrated tanks. The labeled AR was mixed with unlabeled AR carrier and repeatedly crystallized in acetone to a constant specific activity. AR thus purified had the same ultraviolet-visible

spectrum as the authentic sample obtained from Donkersloot (4) and gave only a single spot on thin-layer chromatogram and autoradiograph. AR was quantitated by measuring its absorbance at 453 nm (ϵ 10,500; reference 4).

The labeled ST was purified twice on ChromAR Sheet 500 and Adsorbosil-1 plates developed in benzene:acetone and chloroform:acetone:*n*-hexane, respectively. The zone containing ST was collected and the ST was eluted with acetone. The eluate was concentrated and mixed with an appropriate amount of unlabeled ST (Makor Chemical, Jerusalem, Israel), and was then repeatedly crystallized in benzene to a constant specific activity. The purity of the ST was verified by both ultraviolet spectrum and autoradiography. The concentration of ST was determined by measuring the absorbance at 325 nm (ϵ 13,100; reference 3).

The OP and AF in the acetone extract were separated and purified chromatographically with aluminum oxide column impregnated with 6% oxalic acid. Aflatoxin B_1 was eluted with 35% chloroform in benzene and OP with 20% acetone in chloroform. The pigment thus obtained was further purified by thin-layer chromatography (Adsorbosil-1) plates developed with benzene:ethylacetate:isopropanol:water (25:10:2:1) and then crystallized in 20% acetone in chloroform and quantitated by measuring its absorbance in methanol at 312 nm (ϵ 17,100; Hsieh, Yao, and Reece, Abstr. Pacific Conf. on Chemistry and Spectroscopy, 1973).

Radioactivity measurement. Radioactive samples were pipetted or scraped off the thin-layer chromatography plates into glass vials containing 15 ml of toluene base scintillation fluid (12). The radioactivity was counted in a Packard model 2425 Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). Counting efficiency, as determined by the automatic external standardization method, was in the range of 84 to 88% for all samples measured.

Autoradiogram was prepared by covering a thin-layer chromatography plate with a No-Screen medical X-ray film (Eastman Kodak Co., Rochester, N.Y.) and storing the plates in the dark for 2 to 7 days. The developed autoradiograms were superimposed on the thin-layer chromatography plates to examine the coincidence of spots.

RESULTS AND DISCUSSION

Inhibition of aflatoxins biosynthesis by dichlorvos and accumulation of the orange pigment. The effects of dichlorvos on AF biosynthesis of the 48-h mycelia of *A. parasiticus* in MM and RC media are indicated in Fig. 2. In both media, AF production was terminated and OP began to accumulate in the mycelia and excrete into the media upon addition of 20 ppm dichlorvos. Although AF production was inhibited to the same extent, the yield of OP in RC medium was three times that in MM medium despite the higher glucose concentration in the latter (36 versus 50 g/liter). The lower yield of OP in MM medium, which contained nitrogen, might have been due to the induction of OP metabolizing enzymes, whereas, in RC medium, no such enzymes were synthesized due to the absence of nitrogen source.

When the pregrown cells, further incubated with 20 ppm dichlorvos in MM medium for 24 h, were thoroughly washed with sterile water and reincubated in dichlorvos-free MM and RC media, both containing 50 g of glucose/liter, the relative production of AF B_1 and OP was as shown in Fig. 3. In MM medium where there is ample supply of nitrogen, AF synthesis resumed and increased appreciably, with OP disappear-

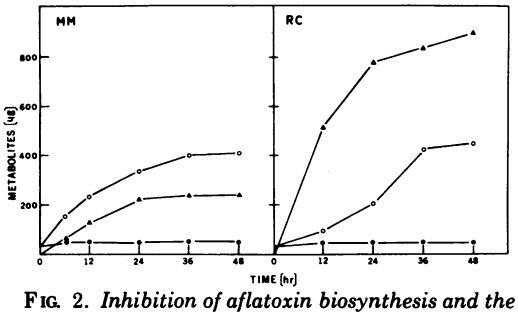

FIG. 2. *Inhibition of aflatoxin biosynthesis and the accumulation of an orange pigment in minimum mineral (MM) and resting cell (RC) media by A. parasiticus treated with 20 ppm dichlorvos at 48 h of growth. Symbols: O, aflatoxin B_1 in dichlorvos-free cultures; ●, aflatoxin B_1 in dichlorvos-treated cultures; ▲, orange pigment in dichlorvos-treated cultures.*

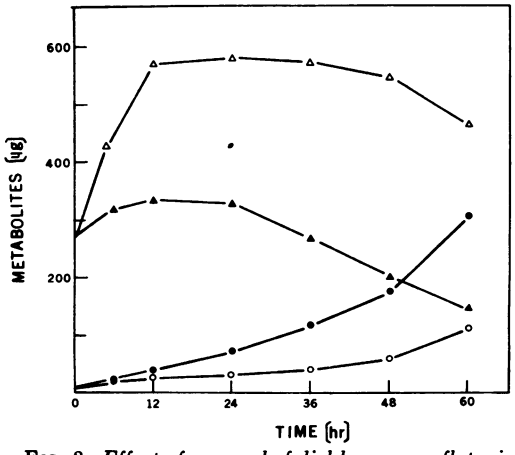

FIG. 3. *Effect of removal of dichlorvos on aflatoxin biosynthesis. Cells previously treated with 20 ppm dichlorvos in minimum mineral (MM) medium for 24 h were washed and resuspended in dichlorvos-free, fresh MM, and resting cell (RC) medium. Symbols: ●, aflatoxin B_1; ▲, orange pigment in MM medium; O, aflatoxin B_1; Δ, orange pigment in RC medium.*

ing from the medium after a slight initial increase. Apparently the repair-synthesis of the dichlorvos-susceptible enzyme in the presence of nitrogen was responsible for the resumption of AF synthesis. In RC medium where no external nitrogen supply was available, AF synthesis resumed at a much slower pace, indicating that dichlorvos inhibition was slowly reversible. The resumption of the enzyme activity became more clear as incubation continued when the cellular level of OP decreased with a faster increase in AF formation.

Identification of the orange pigment. The orange pigment described above was produced using the resting cell culture of *A. parasiticus* supplemented with different amounts of dichlorvos. The effect of dichlorvos concentration on the yield of labeled OP is shown in Fig. 4. The highest incorporation efficiency concurred with 10 ppm dichlorvos. Above that level, the incorporation percentage decreased significantly. The lower yield of OP at higher concentrations of the inhibitor indicated an effect of dichlorvos on another enzyme involved in the earlier portion of the biosynthetic pathway.

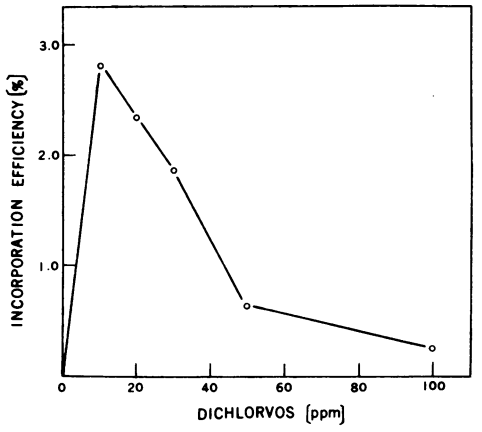

FIG. 4. *Effect of dichlorvos concentration on the efficiency of [1-^{14}C]acetate incorporation into the orange pigment. Cells were incubated in nitrogen-free resting cell medium containing 18 g of glucose per liter.*

We previously reported that the unknown OP gave orange needle-like crystals with a melting point of 219 to 225 C (decomposition) (Hsieh, Yao, and Reece, Abstr. Pacific Conf. on Chemistry and Spectroscopy, 1973). Its ultraviolet spectrum (Beckman DK-2A) showed λ_{max}^{ETOH} 224, 225 (sh), 265, 295, 323 and 480 nm (ϵ_{max} 23,800, 13,000, 14,000, 23,000, 11,300, and 7,250 respectively). The ultraviolet spectrum of OP closely resembled that of versiconol (λ_{max}^{ETO} 224, 255, 265, 295, 322, 460 nm) previously reported by Hatsuda et al. (5). The infrared spectrum (Perkin-Elmer 337) showed bands at 1,675 and 1,625 cm^{-1}, indicating the presence of a nonchelated and chelated anthraquinone carbonyl groups. The mass spectrum (CEC model 21-110 with electron multiplier detector) revealed principal ions at *m/e* of 340, 322, 312, 311, and 297. Other significant ions at 382, 354, and 325 were also noted. At the same time, low-mass ions at 42, 45, and 60 revealed the presence of acetyl group. Based on the physical properties of OP and how it was being produced from cultures of AF-producing strains, there is good reason to believe that OP isolated in this study is probably the same pigment recently isolated by Schroeder et al. (17). Our data indicated that OP is probably an acetyl derivative of a versiconol-type compound. Analytic information obtained thus far is not sufficient to allow an unequivocal identification of OP. Further studies are in progress for its structural elucidation.

Conversion of orange pigment into aflatoxin B_1. The incorporation of [^{14}C]OP and [1-^{14}C]acetate into AF B_1 by wild-type *A. parasiticus* is indicated in Table 1. After repeated thin-layer chromatography purification, AF B_1 retained 13.7% of the radioactivity from the [^{14}C]OP, but less than 1% of the activity from [^{14}C]acetate, despite the fact that the concentration of OP (0.06 mM) used in the experiment was much lower than that of acetate (8.0 mM). The efficient incorporation of radio-

TABLE 1. *Conversion of ^{14}C-labeled orange pigment and [1-^{14}C]acetate into aflatoxin B_1 by A. parasiticus*[a]

^{14}C-labeled precursor	Net radioactivity uptake (10^6 dpm)	Sp act of precursor (10^6 dpm/μmol)	Aflatoxin B_1[b]		
			Amount (μmol)	Radioactivity (10^4 dpm)	Incorporation efficiency (%)
Orange pigment	0.586	2.03	0.486	0.80	13.7
Sodium acetate	88.5	1.1	0.404	8.28	0.94

[a] Portions of 1.5-g mycelial pellets grown in minimum mineral medium for 48 h were washed and resuspended in 10 ml of resting cell medium containing 300 μmol of glucose and 0.1 ml of ^{14}C-labeled precursor in methanol. Results were the mean of triplicate samples.

[b] Aflatoxin B_1 was purified in three different thin-layer chromatography systems.

activity from OP over that from acetate, a known precursor of AF (10), in the nitrogen-free resting cell medium indicates that OP was converted into AF B_1 without undergoing major degradation and is probably an intermediate in AF biosynthesis.

Conversion of sterigmatocystin and averufin by dichlorvos-treated cultures of A. parasiticus. Lin et al. (12) and Hsieh et al. (9) have shown that AR and ST could both be converted into AF B_1 by the resting mycelia of wild-type *A. parasiticus*. The results in Table 2 indicate the effect of 10 ppm of dichlorvos on the incorporation of labels from acetate, AR, and ST into AF B_1 in the resting cell culture. The incorporation efficiency for [1-^{14}C]acetate into AF B_1 by the untreated culture indicates that the mycelia used had normal AF synthetic activity. Incorporation of [^{14}C]AR into AF B_1 was completely inhibited by dichlorvos, but incorporation of [^{14}C]ST was unaffected, indicating that the enzyme system responsible for converting AR into ST was the site of dichlorvos action. In the presence of dichlorvos, the biosynthetic pathway terminates at the formation of OP, as indicated by the incorporation into OP of ^{14}C from both sodium acetate and AR. Therefore, OP is an intermediate in the conversion of AR to ST, and its structure should give useful information as to the mechanism of the development of the bisfuran ring structure in AF B_1.

Thus, dichlorvos at low concentrations inhibits a specific enzymatic step in AF biosynthesis and effects the accumulation of a biosynthetic intermediate of AF. The step of inhibition resides beyond AR and preceding ST on the AF biosynthetic scheme. Our experimental results also revealed the involvement of another C_{20} compound, i.e. OP, in addition to AR ($C_{20}H_{16}O_7$), in the biosynthesis of AF.

The experimental evidence accumulated thus far does not seem to provide a definite answer to the pending question of how the bisfuran structure was developed in the biosynthesis of ST and AF. Based on the labeling pattern of AF B_1 derived from [1-^{14}C]acetate and [2-^{14}C]acetate and the uniform label density throughout the molecule (Fig. 1), Biollaz et al. (2) advanced a hypothetical pathway involving a single C_{18}-polyketomethylene compound as intermediate; the bisfuran ring structure was proposed to be derived from endoperoxidation of a terminal benzene ring in a hypothetical polyhydroxynaphthacene or polyhydroxybenzanthracene intermediate. This scheme does not accommodate AR as an intermediate, which has been experimentally shown to be convertible in vivo into AF B_1 (9). Therefore, the C_{18}-polyketide theory is highly questionable. Recently, Thomas (18) postulated that AR is an intermediate in the biosynthesis of ST and AF and that the C_4-bisfuran structure is derived from the C_6 side chain of AR through an acetylated furan structure as intermediate. Unfortunately, this acetylated furan structure was not found in our OP or the recently found versiconal acetate (17).

Alternatively, it has been proposed that ST and AF are synthesized in vivo from a fusion of two preformed polyketide units, namely C_4 and C_{14} moieties (6, 7). The "two-unit theory" was supported by the difference found in the labeling density of the bisfuran and the xanthone moiety of the [^{14}C]ST molecule synthesized from [^{14}C]acetate (7), and also by the efficient incorporation of acetoacetate into AF B_1 (6).

TABLE 2. *Incorporation of ^{14}C-labeled sodium acetate, averufin, and sterigmatocystin into aflatoxin B_1 by A. parasiticus treated with 10 ppm dichlorvos*[a]

Precursor[b]	Aflatoxin B_1			Orange pigment	
	Amount (μmol)	Radioactivity (10^4 dpm)	Incorporation efficiency (%)	Radioactivity (10^4 dpm)	Incorporation efficiency (%)
Sodium acetate	0.392	8.28	0.94	NR	
Sodium acetate + dichlorvos	0.024	NR[c]		10.4	1.18
Averufin	0.640	10.38	49.4	NR	
Averufin + dichlorvos	0.029	NR		16.68	79.4
Sterigmatocystin	0.651	16.25	65.0	NR	
Sterigmatocystin + dichlorvos	0.528	17.87	71.5	NR	

[a] *A. parasiticus* grown for 48 h in minimum mineral medium was transferred to 10 ml of resting cell medium containing 300 μmol of glucose. Results were the mean of duplicate samples.

[b] Amount of ^{14}C-labeled precursors added: sodium acetate (1.1×10^6 dpm/μmol), 80 μmol; averufin (4.8×10^5 dpm/μmol), 0.438 μmol; sterigmatocystin (5.9×10^5 dpm/μmol), 0.423 μmol.

[c] NR, No radioactivity.

The substitution of the C_6 side chain of AR with an acetoacetate-like C_4 unit to form a versiconol-like structure would provide an easy explanation of how the bisfuran ring system in the molecules of ST and AF is developed. More experimental work needs to be done to further understand the origin and mechanism of formation of the fungal bisfuranoid structure.

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