Evidence for the Metabolic Detoxification of n-dodecylguanidine Acetate by Ungerminated Macroconidia of Fusarium solani f. sp. phaseoli

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ABSTRACT

Macroconidia of *Fusarium solani* f. sp. *phaseoli* sorbed dodine-^{14}C from aqueous solution and subsequently released a ^{14}C-labeled material that differed from the parent molecule. When 10 μg of dodine-^{14}C was supplied to 10^7 conidia/ml, 63% of the dosage was sorbed in 30 min. After 96 min of exposure, however, two-thirds of this had been altered and released. The labeled compound that was released was much less toxic than dodine, and differed in solubility and chromatographic properties. The process apparently was dependent upon metabolic action, since it did not occur in boiled conidia, and was delayed in cycloheximide-treated spores and in spores held at 0 C. The rate of release of the detoxified material was temperature-dependent. Metabolic detoxification of dodine may be the mechanism by which *F. solani* f. sp. *phaseoli* is resistant to the action of dodine. Phytopathology 60:350-354.

n-Dodecylguanidine acetate (dodine) is taken up rapidly by most fungi that have been tested, and, once bound to conidia, cannot be washed off by water (4, 6, 8, 9). Furthermore, dodine-^{14}C bound to conidia in an aqueous suspension would not exchange with added, unlabeled dodine. Only when treated with an agent known to disrupt the plasmalemma of cells could dodine be released from the cells (6). These reports and the rate of uptake of dodine by *Neurospora crassa* led Somers & Pring (9) to suggest that dodine binds by an ionic mechanism to carboxyl and phosphate groups on the cell wall.

Such permanent attachment is not always the case. A study of the toxicity of dodine in two sensitive and two resistant fungi showed that the sorption of dodine-^{14}C by macroconidia of *F. solani* increased to a maximum value, then decreased, indicating the release of a ^{14}C-labeled compound into the suspending liquid (3). The release was in marked contrast to results of earlier studies involving other organisms where no release of the dodine occurred once it was bound to fungal cells. Since dodine itself is not readily released from the surface to which it is absorbed, some modification may have occurred in the molecule. No previous report of such modification has appeared. Somers (8) reported that dodine sorbed by conidia of *N. crassa* exposed for 30 min to a sublethal dosage level and then extracted by boiling methanol was identical with the parent compound. Curry (5) reported that approximately 0.2% of a dodine-^{14}C preparation applied to apple leaves appeared in apple fruit as degradation products. The data suggested that the guanidine moiety was separated from the dodecyl moiety, and was incorporated into the cell's normal pools of amino acids, peptides and closely related compounds. The low percentage of the applied ^{14}C which appeared in the fruit (less than 0.2%) as compared to the reported radiopurity of 95% suggests that the impurities in the initial preparation could have been the source of the "degradation products".

The following study examined the release of radioactive materials from dodine-^{14}C-treated F. *solani* f. *phaseoli* macroconidia to determine whether the released material was dodine or material(s) resulting from an alteration of the dodine molecule. The rates of uptake and release were analyzed to determine the nature of the processes involved.

MATERIALS AND METHODS.—Dodine-^{14}C, having a specific activity of 125 μc/mmole, was synthesized by refluxing a solution of dodecyclamine, cyanamide-^{14}C, acetic acid, water, and isopropanol as described for the first part of the synthesis of allylol-substituted alkyl or alkaryl guanidines (1). Purification and determination of radiopurity of the dodine-^{14}C preparation have been reported earlier (2).

The descending paper chromatographic procedures used to identify radioactive compounds in this study were those reported by Curry (5). The solvent system was pyridine:isoamyl alcohol:water at a ratio of 80:40:70. Whatman No. 1 paper strips 1.5 inch wide were used. After the strips had been developed and dried, they were scanned for radioactivity with an Atomic Accessories Inc. Model RSL 160 open-window-strip scanner. Only 75% of the ^{14}C in the preparation was present in dodine-^{14}C; however, the radioactive materials sorbed by conidia of four different fungi (3) from the crude preparation, when extracted and chromatographed on paper, moved to a single peak at or near the same R_F as the radioactive dodine-^{14}C. This peak coincided with the fungitoxic area of a similarly processed chromatograph of unlabeled dodine. Thus, while ^{14}C-labeled impurities did exist in this dodine-^{14}C preparation used, they were not sorbed by conidia used in this study, and hence did not interfere with the studies reported.

For uptake analyses, conidia at a concentration of 10^7/ml were treated in a continuously agitated solution of dodine-^{14}C in distilled water. Aliquots were removed and processed as described previously (3). Measurement of radioactivity as well as production and preparation of conidial suspensions of *Fusarium solani* f. sp.

phaseoli (Burk.) Snyd. & Hans., and *Colletotrichum orbiculare* (Berk. & Mont.) v. Arx were performed as described previously (3).

The fungitoxicity of the radioactive materials released from *F. solani* macroconidia after treatment with dodine-^{14}C was determined as follows: Macroconidia were treated at 10 μg dodine-^{14}C/10^7 conidia/ml water for 30 min. The conidia were then collected on a Millipore filter, washed with several 2-ml volumes of distilled water, and suspended in distilled water for 1 hr. During this period release of the ^{14}C-labeled material occurred. The spores were then removed by centrifugation and the supernatant containing the altered material collected. The sorbed material was obtained from a similar lot of spores exposed to dodine and washed in the manner described above and then suspended immediately in methanol (MEOH). Both extracts were dried and the residue of each dissolved in methanol for bioassay to determine fungitoxic activity.

The biological activity of the two extracts was compared on a basis of equivalent radioactivity (decompositions per min., dpm). Aqueous solutions containing 4, 8, 16, 32, and 64 μg dodine-^{14}C/ml were prepared. Dilutions of the two extracts described above were prepared so as to contain equivalent dpm levels. Drops containing 0.02 ml of each solution were then applied to designated loci on glass slides, and the MEOH evaporated. An equal volume of a suspension containing 1×10^4 conidia of *F. solani* was then added to the treated loci, and the slides were incubated for 10 hr in a moist chamber. Tests were set up in triplicate, and 100 spores counted/locus. All experiments reported were repeated at least once, and the data reported is representative of that obtained.

RESULTS.—A comparison of the fungitoxicity of the sorbed and of the released material with that of dodine indicated that both were much less toxic than dodine (Table 1).

When spores were exposed to an amount of released materials equivalent in radioactivity to 64 μg dodine-^{14}C/ml, inhibition was only one-fourth that caused by 16 μg dodine-^{14}C. The fact that both test materials were much less toxic than dodine-^{14}C indicated that a detoxification had occurred before the radioactive materials were released from the conidia. Whether the fungistatic activity detected was a property of the altered compound or due to residual dodine was not apparent.

TABLE 1. Germination of conidia of *Fusarium solani* exposed to dodine-^{14}C and to the products sorbed to and released from macroconidia of *Fusarium solani* treated with dodine-^{14}C

Concn. of test material (μg/ml) or dpm equivalent[a]	Germination of conidia after exposure to: Dodine-^{14}C	Product sorbed by *F. solani*	Product released in distilled water
4	85[b]	97	95
8	19	93	95
16	0	94	
32	0	90	88
64	0	75	74

[a] μg/ml dodine and solutions of the two products having equivalent decompositions per min.
[b] % Germination.

The R_F of the labeled material released by macroconidia treated with dodine-^{14}C was chromatographed by procedures described above. Peaks of radioactivity occurred at R_F 0.91 on the paper strips to which reaction products had been added. Dodine had an R_F of 0.93 when chromatographed simultaneously on a separate strip. Verification of this shift in R_F was checked by chromatographing strips containing (i) parent dodine-^{14}C; (ii) detoxified materials; and (iii) a combination of the two. After development, the last 35-mm section of each of the three strips was cut into seven 5-mm sections, and each section was placed in a separate scintillation vial. The vials were assayed for radioactivity (Table 2).

The R_F for the released material calculated from Table 2 was 0.90, while that for dodine was 0.95. The combination of the two resulted in a broad area of radioactivity encompassing both peaks.

Assurance that this small difference in R_F was reproducible was obtained by analyzing data from a series of simultaneous chromatographs of (i) untreated dodine-^{14}C; (ii) radioactive materials released by conidia of *F. solani;* and (iii) material extracted from *C. orbiculare* conidia that had been treated in a manner similar to that for *F. solani* as described previously (2, 3). The ^{14}C-labeled material extracted from *C. orbiculare* conidia was chromatographed to determine whether a comparable alteration in the chromatographic characteristics of dodine-^{14}C occurred due to the binding and/or extraction. The R_F's of radioactive areas on the completed chromatographs were measured, averaged, and compared statistically, using the t-test for paired observations. The means were 0.91 for the ^{14}C-labeled material released by conidia of *F. solani,* and 0.95 for that extracted from conidia of *C. orbiculare* and for dodine-^{14}C. The probability that the differences between these means was due to chance was less than 1%. Methanol extracts of untreated macroconidia had no effect on the chromatographic characteristics of dodine-^{14}C. Although the difference between R_F's was slight, use of paired chromatograms (released ^{14}C materials on one and parent dodine-^{14}C on the other) allowed objective determination of the existence of a labeled product other than dodine. Extensive efforts to find other combinations of solvents to more effectively separate dodine-^{14}C or the released material on the chromatograms failed to produce one superior to the pyridine:isoamyl alcohol:water system used throughout these studies.

The solubility characteristics of the detoxified material served to further indicate that a change in the dodine molecule had occurred. Dodine-^{14}C and the detoxified material were normally extracted from dried filtrates and spore suspension with MEOH. However, when several MEOH solutions containing detoxified materials were concentrated, a precipitate containing the ^{14}C-labeled materials that was soluble in diethyl ether was formed. Later, a residue containing detoxified

TABLE 2. Distribution of ^{14}C-labeled compound in paper strip chromatograms of dodine-^{14}C, the detoxified product, and of a mixture of the two materials.

	Chromatogram section[a]						
	1	2	3	4	5	6	7
Dodine-^{14}C	36[b]	73	86	687	1,875	2,713	1,307
Detoxified product	289	372	942	1,076	1,028	746	
Combination	266	474	560	1,284	1,345	2,048	1,212

[a] Successive 5-mm sections of the terminated 35 mm of simultaneous paper strip chromatograms.
[b] Decompositions per min (dpm).

material was extracted twice with methanol, dried, then extracted with diethyl ether. There was appreciable radioactivity in the ether extract which chromatographed to a single peak at R_F 0.89. Since dodine is insoluble in diethyl ether, the ether solubility of the detoxified materials suggested that an alteration had taken place which made the product less polar than parent dodine.

The effect that lethal dosages of dodine would have on the uptake of that chemical and the subsequent release of altered materials by conidia of *F. solani* were determined. Four dosages of dodine-^{14}C ranging from 10-40 μg/10^7 spores were used. Aliquots of the suspensions were removed at various time intervals. The germination inhibition after 96-min exposure at each dosage was determined and is included at the right of each curve (Fig. 1-A).

The maximum amounts of dodine-^{14}C sorbed on the conidia were 53, 30, 40, and 41% of the initial 10, 20, 30, and 40 μg/ml dosages, respectively. The percentage of maximum sorbed radioactivity which was released by 96 min at each of the four dosages was 86, 69, 23, and 14%, respectively. The percentages released were also determined after 3 hr of exposure at the three higher dosages, and found to have increased to 90, 67, and 64% of the peak retention, respectively. The fact that release continued although 99% of the conidia failed to germinate when placed in suitable medium after an exposure to 40 μg dodine/ml for 96 min, suggested that germinable conidia were not required for this step.

The reduction in the amount of radioactivity released after 96 min of exposure at increased dosages may have represented either an inhibition of the detoxification procedure as a result of the toxic action of dodine or a masking of release due to continued uptake of dodine-^{14}C. To differentiate between these possibilities, the filtrates taken after 32-, 48-, 64-, 80-, and 96-min exposure from each of the dosages were pooled and dried, and the residue was dissolved in MEOH and chromatographed. The R_F's of the radioactive materials present in the filtrates at the three lower dosages were 0.89-0.92, while that at the 40 μg dosage was 0.94. This represented unsorbed and unchanged dodine-^{14}C. After 180-min exposure, the radioactive compounds present in filtrates from tests at all four dosages had R_F's of 0.91. Thus, the highest dosages delayed the release of radioactive compounds from the dodine-^{14}C-treated conidia.

The decreased detoxification at near lethal doses suggested that metabolic processes were involved with the alteration of dodine. As a test of the requirement for viable conidia for detoxification of dodine, a tube with a suspension containing 2×10^7 conidia of *F. solani*/ml was placed in boiling water for 7 min; the spores were then washed and exposed to 20 μg dodine-^{14}C/10^7 conidia/ml (Fig. 1-B). Metabolically inactive spores not subjected to the harsh treatment of boiling water were provided by exposing viable conidia to 20 μg dodine-^{14}C/ml in an ice bath (Fig. 1-B). Sorption of dodine by boiled conidia was rapid, with the maximum uptake occurring within 4 to 8 min of exposure. There was no evidence of release of radioactivity. Radioactive compounds extracted from the boiled conidia after 96 min of exposure to dodine-^{14}C had an R_F of 0.95, demonstrating that boiled conidia did not modify the dodine molecule.

Initial uptake of dodine-^{14}C at 0 C by *F. solani* macroconidia was rapid, and was completed by the end of 4-min exposure. Little additional uptake was measured during the subsequent 96 min; however, additional uptake did occur between 96 and 180 min of exposure to dodine-^{14}C at 0 C. The radioactive materials extracted from conidia after 30 and 90 min of exposure had an R_F of 0.94. These data indicate that detoxification requires viable conidia. Although detoxification occurred at 0 C, it was very slow and delayed, possibly due to the slight warming of the mixture that occurred each time samples were removed.

The possible requirement for protein synthesis in the process of detoxification was determined by pretreating conidia with 50 μg cycloheximide/2×10^7 conidia/ml for 2 hr. At this concentration, cycloheximide decreased the subsequent germination of treated and then washed conidia by about 10%, as compared with conidia soaked in distilled water and otherwise handled similarly. Conidia held in water for 2 hr served as the control. After washing with distilled water, both groups of conidia were exposed to 20 μg dodine-^{14}C/10^7 conidia/ml (Fig. 1-C).

Maximum uptake of dodine-^{14}C by the conidia pretreated with cycloheximide occurred at 64 min, while maximum uptake by conida held in distilled water was at 32 min of exposure. Germination of the cycloheximide-pretreated conidia was inhibited by 86% after 128 min of exposure to the dodine-^{14}C solution, while a similar value for those not pretreated was 60%. The principal peak of radioactivity of material extracted from the cycloheximide-pretreated conidia after 128 min of exposure to dodine was at R_F 0.95. On this chromatogram, however, the main peak was preceded by a smear of unresolved radioactivity which began at approximately R_F 0.6. A chromatogram of a cycloheximide dodine-^{14}C mixture did not possess this smear.

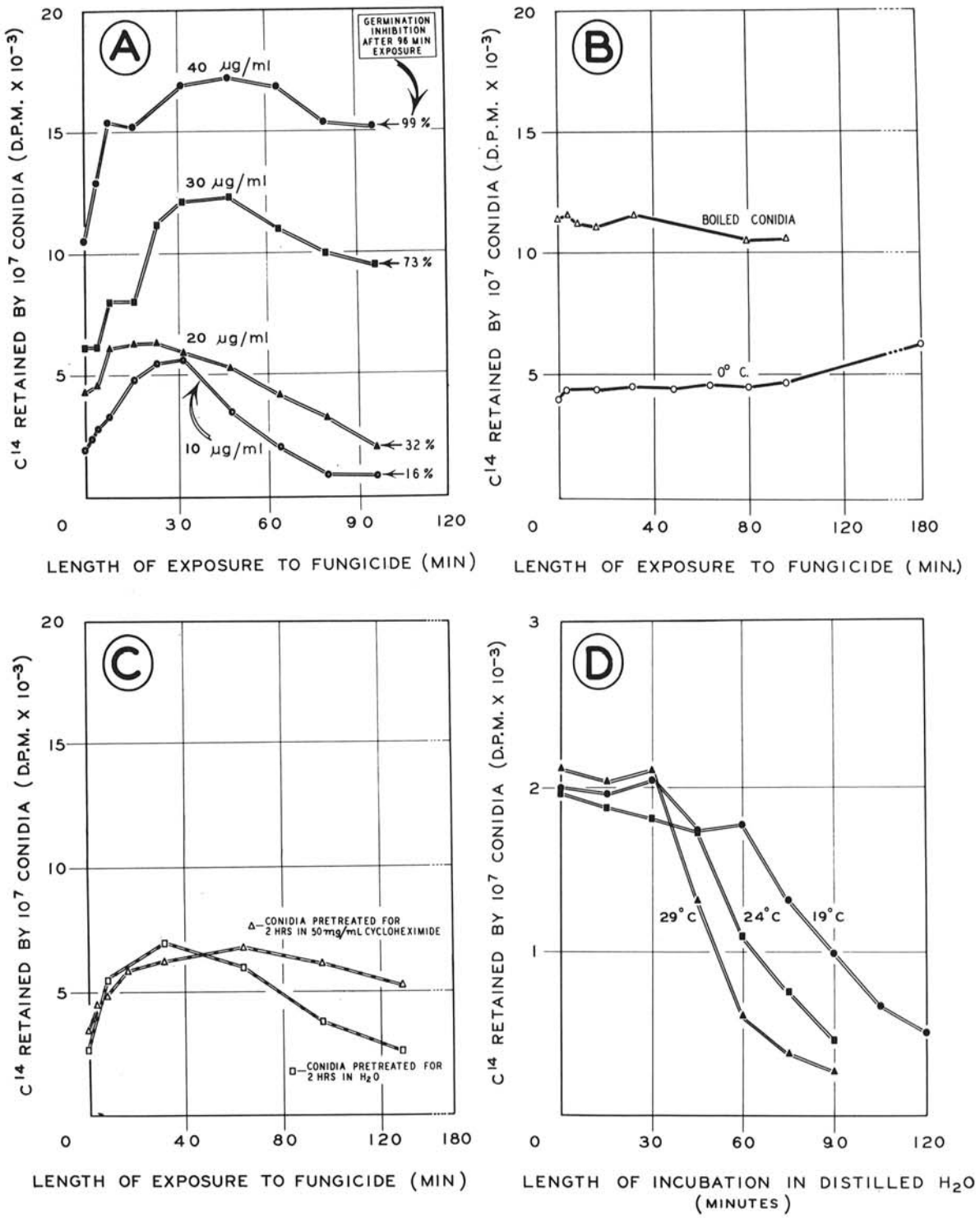

Fig. 1. ^{14}C retained by conidia of *Fusarium solani* f. sp. *phaseoli*. **A)** Exposed to four dosages of dodine ^{14}C over a period of 96 min. **B)** Rendered metabolically inactive by boiling before treatment or by exposure and incubation at 0 C. **C)** Pretreated for 2 hr with 50 mg/ml cycloheximide, then exposed to 20 µg dodine/10^7 spores/ml. **D)** Incubated at different temperatures after initial exposure to 10 µg dodine-^{14}C and 10^7 conidia/ml, and subsequent washing to remove excess labeled dodine at 0 C.

These data suggested that conidia pretreated with cycloheximide detoxified dodine less readily.

The rate of release of sorbed material was determined at 19, 24, and 29 C. Conidia were all exposed at 0 C to 10 μg dodine-$^{14}C/10^7$ conidia/ml for 1.5 hr. After exposure, the conidia were washed at 0 C, and equal aliquots were placed into equal volumes of distilled water in each of three Erlenmeyer flasks continuously agitated in a bath at 0 C. The "zero time" samples were removed from each of the three aliquots of the treated conidia before incubation at the three temperatures. The similarity of the initial counts of these samples indicated the uniformity of sorbed dodine-^{14}C in the three test groups. One flask was then placed in a water bath at each temperature, and aliquots were removed from the flasks at 15-min intervals, washed, and counted as described previously.

There was a definite effect of temperature on the time and the rate of release of ^{14}C-labeled material (Fig. 1-D). The release of radioactive compounds by conidia held at 29 C began 30 min before it did at 19 C. In addition, the rate of release increased with temperature. The Q_{10} calculated from the rates of release of radioactivity from the conidia held at 29 and 19 C was 2.18, and is in the range expected for metabolic reactions.

Discussion.—Ungerminated macroconidia of *Fusarium solani* f. sp. *phaseoli* metabolically altered dodine to a compound(s) much less toxic than the parent material. Whether this is a partial or a complete detoxification will not be known until the products are separated and identified. The toxicity present in the solution of released material may be from residual dodine-^{14}C, or it may be a characteristic of the new compound(s).

The release of radioactive materials after detoxification by the conidia suggested that the alteration in the chemical structure of parent dodine had resulted in the loss of its tenacious binding properties. The released radioactive material was not volatile, had different solubility properties, and had an R_F value that differed slightly from that of dodine. The solubility change suggested that the alteration in structure must have made the guanidine moiety less polar.

The detoxification of dodine by *F. solani* conidia was apparently a characteristic of viable conidia, since boiled conidia were unable to detoxify dodine nor to release any sorbed radioactivity. The increased sorption of dodine (1.5-2.0 times) by boiled conidia suggested that the boiling may have exposed additional binding sites. Three other treatments that would inhibit all or part of the metabolic system of the conidia reduced the rate of release and increased the incubation period required for detoxification. In the most conclusive of these tests, conidia were exposed to dodine at 0 C. Detoxification only occurred after 180 min of exposure, as compared with the 30 min required at room temperature.

Pretreatment with cycloheximide delayed detoxification and increased the time required for maximum uptake. This suggested that detoxification was metabolic and required protein synthesis. We assume that the mode of action of cycloheximide on *F. solani* conidia is similar to that on yeast cells (7), and that the protein synthetic processes of the macroconidia were affected by the antibiotic treatment.

Still other evidence supporting the metabolic nature of detoxification came from the analysis of the effect of temperature on the release of detoxified materials. The conidia held at the higher temperature began releasing radioactivity 30 min earlier than did those held at the lower temperature.

While the release could be either (i) a physical process (diffusion from internal sites, etc.) or (ii) a metabolic process, the Q_{10} calculation for the rate of release (2.18) would seem to eliminate physical release from external sites as an explanation. These and earlier data were not precise enough to determine whether detoxification and release occur simultaneously or in succession.

Despite the fact that the release of the detoxified material occurred from conidia prevented from germinating by dodine, the process might be enzymatic, since Brown & Sisler (4) and Tweedy (10) have reported that lethal dosages of dodine did not inhibit the activity of various enzymes in in vitro or in vivo analyses.

The metabolic detoxification of dodine by *Fusarium solani* f. sp. *phaseoli* may be a mechanism of resistance to dodine. However, more information is needed to establish (i) the nature of the detoxification process in terms of the enzyme systems involved with the process, and (ii) the products resulting from detoxification in order to understand the relationship between detoxification and resistance to dodine.

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