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Abnormal cilia in a male-sterile mutant mouse

John H.D. Bryan*
Department of Zoology, University of Georgia Athens, Georgia 30602 USA

Summary. In mice homozygous for the mutation hydrocephalic-polydactyl (*hpy*) ciliary axonemes from tracheal, oviducal, and ependymal lining cells showed a variety of abnormalities. Defects included: a deficiency of inner dynein arms, extra central tubules, a displacement of one outer doublet and/or the central tubules, and double axonemes. More than one kind of defect was seen in some axonemes. None of the types of defects observed in mutants were encountered in equivalent samples from non-mutant littermates. Except for the most common defect, the deficiency in dynein arms, which occured to about the same extent (approximately 34%) in all three tissues, there were marked variations in frequency among the tissue types with respect to the other defects. In general, defects such as central tubule anomalies, displaced tubules, and double axonemes occured with the highest frequencies in axonemes from tracheal epithelial cells and with the lowest frequencies in samples of oviducal epithelium. Fused cilia were seen only in ependymal cell samples. Some of the defects encountered were common to sperm flagella axonemes while others appeared restricted to somatic tissues, suggesting, perhaps, each tissue type may exert its own modulating influence on the expression of the mutant gene.

Key words: Axonemal defects – Cilia – Mouse – Male-sterile mutant

Introduction

The recessive mutation *hpy* (hydrocephalic-polydactyl) on chromosome of 6 of the mouse (Hollander 1976) produces as one manifestation of its pleiotropic character complete sterility in the male. In homozygotes the organization and development of the axoneme and associated flagellar components

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is profoundly affected resulting in a complete absence of intact sperm tails (Bryan 1977 and 1981). Although axonemal development is initiated on schedule the resulting structures are not stable and do not persist throughout the period of sperm formation. Only very rarely were any semblances of axonemes encountered in the more-advanced spermatids. Furthermore, various structural defects were noted in the developing flagellar axonemes and certain of them resembled those reported by others in male-sterile human patients with the immotile cilia syndrome (for details see Bryan 1981 and 1983). In contrast to males, mutant females are fertile but they are unpredictable breeders; at best a female may produce three medium-sized litters (6–8 offspring per litter) during her reproductive lifetime. This raises the question of whether or not the cilia of the reproductive tract may be defective and, if so, if they are responsible for the reduction in reproductive performance (c.f., Afzelius et al. 1978). In both sexes non-obstructive hydrocephalus develops post-natally (Bryan 1977); conceivably this additional manifestation of the mutant state could stem from defective ciliary activity. Thus, in view of the foregoing findings it was of considerable interest to determine whether the microtubule-related defects were restricted to the male germ-line or whether they also appeared in male somatic tissues, and also whether similar defects were present in the cilia of females. Both qualitative and quantitative aspects are of importance in this connection since they would bear on the apparent discrepancy between the observed reproductive performance of mutant females and that predicted on the basis of the previous spermiogenetic investigations. This paper reports in detail the results of my ultrastructural investigations of ciliary axonemes from the trachea, oviduct, and ependyma from *hpy/hyp* mice. As will become evident, certain of the findings strongly paralleled those reported for human patients with the immotile cilia syndrome suggesting that this mutant type might serve as a useful animal model for the human condition; this question has been examined in detail elsewhere (Bryan 1983). As will be seen, while the findings indicate that certain of the defects are common to flagellar axonemes and to those of cilia from each of the tissue types examined, other defects noted in ciliary axonemes were not seen in spermiogenetic cells. An additional complication stems from findings indicative of variations between cilia from the different tissues examined with respect to the frequencies of the different kinds of axonemal defects. Thus, the situation proved to be rather more complex than originally anticipated; suggesting that ciliary axonemes of mutants are inherently more stable than their counterparts in spermatids and, perhaps, that each tissue type may exert its individual modulating influence on the expression of the mutant gene. A preliminary report of part of this work has been presented elsewhere (Bryan and Chandler 1978).

Materials and methods

Several pairs of adult mutant and non-mutant littermates were sacrificed and samples of trachea, oviduct, and ependyma removed and processed in various ways. Living cell preparations of tracheal epithelium were used for studies of ciliary activity. Small sections of trachea

were removed into isotonic saline or Hank's balanced salt solution (HBSS) and small pieces of the epithelial lining dissected free of associated tissue and mounted in drops of fresh HBSS or saline. Coverslips were sealed with paraffin wax and the preparations were examined with the phase-contrast microscope. For scanning electron microscopy (SEM), tissue samples were rinsed well in normal saline, fixed in 2% glutaraldehyde in 0.2 M cacodylate buffer pH 7.2 for 45 m, washed in 0.1 M cacodylate buffer pH 7.2 containing 5% sucrose, and post-fixed for 1 h in 2% osmium textroxide in 0.2 M cacodylate buffer. The samples were then washed well in the same buffer, dehydrated through a graded ethanol series and dried by the critical-point method. After mounting on SEM studs the samples were coated with gold (or gold-palladium) in a vacuum evaporator and examined in a Kent-Cambridge Mark IIA SEM. For transmission electron microscopy (TEM) some samples were fixed and processed as described previously (Bryan and Wolosewick 1973). Others were fixed for 1–2 h in 1.5% glutaraldehyde-1.0% tannic acid in 0.1 M cacodylate buffer (after the method of Kuhn and Engleman 1978), washed in the same buffer and post-fixed in osmium tetroxide (as above). After fixation the samples were washed in distilled water, stained en-bloc in 0.5% aqueous uranyl acetate for 30 m and washed overnight in buffer. Further processing was as described earlier (Bryan and Wolosewick 1973). Grids were examined in either a Philips 200 or Siemens Elmiskop 101 at 80 Kv. Fields containing cilia were selected at random, axonemes were classified as normal or abnormal, and counts were made to determine the relative frequencies of the various types of abnormalities encountered.

Results

As exemplified by the oviduct (Figs. 1, 2) the epithelium from each of the three sites (oviduct, trachea, ependyma) appeared to possess normal numbers of ciliated cells. Similarly, each ciliated cell was well-endowed with cilia (Figs. 2, 3). It was difficult to distinguish between living cell preparations from mutant and non-mutant animals on the basis of their respective levels of ciliary activity. Evidence favoring normal ciliary activity was also seen in TEM preparations (Fig. 4). None of the samples from any of the three tissues from mutants revealed any indication of axonemes undergoing dismantling. Following fixation with glutaraldehyde-tannic acid, the walls of both the central-pair tubules and the A-subtubules of outer doublets could be seen to be composed of 13 protofilaments while the walls of B-subtubules contained 10 unshared protofilaments. When present extra central tubules (singlets) were also composed of 13 protofilaments. Various abnormalities were, however, encountered in axonemes from all three tissue types. It should be noted that no cells were discovered in which all the cilia were judged to be abnormal. Instead, normal and abnormal cilia were intermixed on a given cell. The distribution of cilia with abnormal axonemes was heterogeneous. In some instances they were rather widely scattered while in others several abnormal axonemes could be recognized in the same general vicinity (Fig. 5). The most commonly encountered defect was a deficiency of dynein in that inner arms were reduced or lacking; in the oviduct occasional axonemes appeared to lack both inner and outer arms. The frequency of dynein-deficient cilia varied somewhat from one tissue type to another, being lowest in ependyma and highest in oviduct (Table 1). The second most frequent class of abnormality concerned the number and disposition of axonemal tubules. While occasional axonemes lacking one or both members of the central-pair tubules were seen, the presence of up

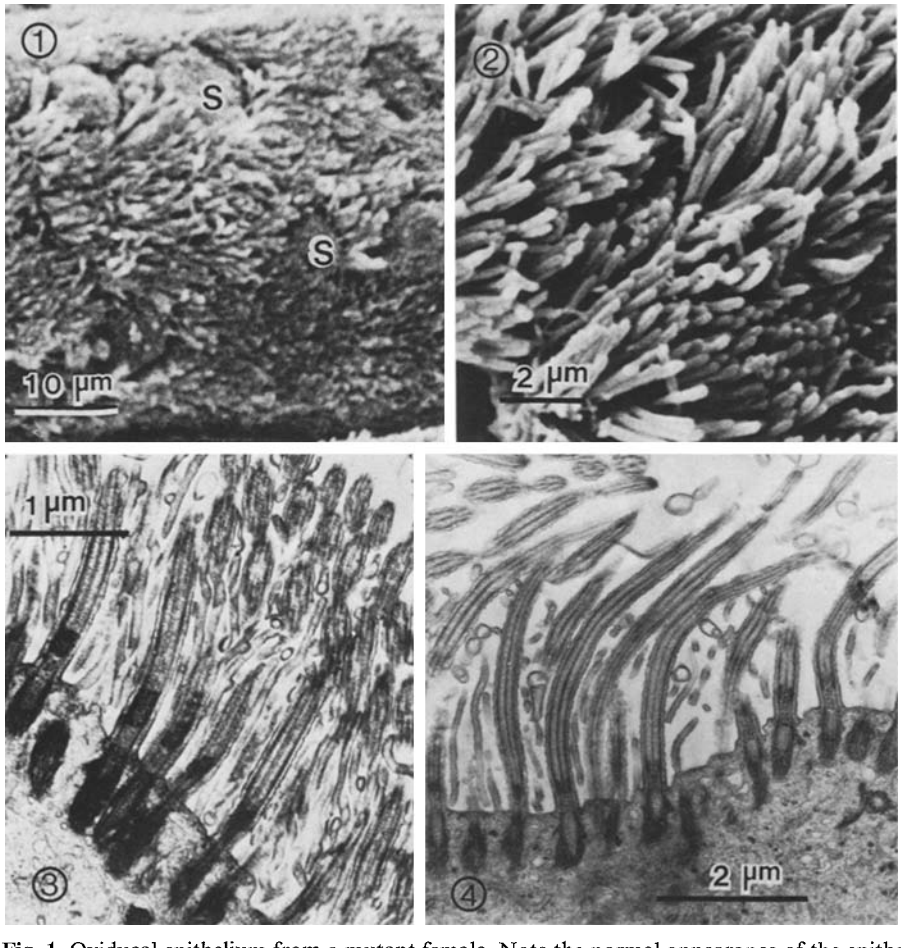

Fig. 1. Oviducal epithelium from a mutant female. Note the normal appearance of the epithelium. Secretory cells (*S*) are intermixed with ciliated cells as in the epithelium from non-mutant animals. Unless otherwise indicated the magnification represent 0.2 µm

Fig. 2. A portion of the same sample as in Fig. 1, seen at higher magnification showing the typical form and distribution of the cilia

Fig. 3. A TEM preparation of tracheal epithelium from a mutant showing the normal distribution of cilia on the cell

Fig. 4. A similar preparation of oviducal epithelium from a mutant. Note that the cilia give the appearance of beating at the time of fixation

to three extra central singlet tubules (9+3, 9+4, 9+5 patterns) was much more common (Figs. 5, 6). Such anomalies were four to five times more frequent in samples from the trachea than in those from the ependyma and oviduct (Table 1). Similarly, the frequency of axonemes exhibiting a displacement of an outer doublet and/or the central tubules was much higher

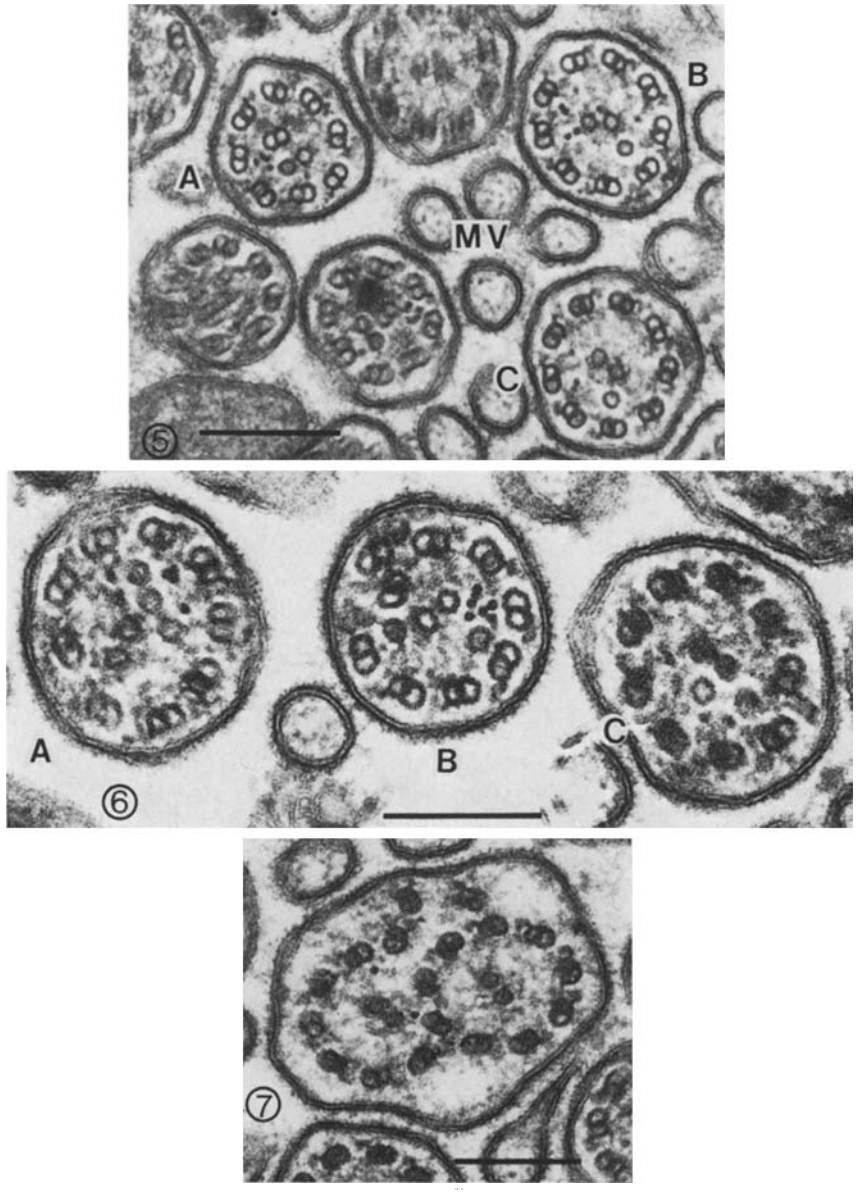

Fig. 5. A preparation of mutant tracheal epithelium. Note the typical appearance of microvilli (*MV*) interspersed among the cilia. One cilium (*A*) shows a displacement of one outer doublet (8−1+2 pattern); the cilium at B exhibits an extra central tubule (9+3 pattern) as does its neighbor (*C*). Note also a general absence of inner dynein arms

Fig. 6. A tracheal sample from a different animal showing similar axonemal anomalies. One cilium (*A*) exhibits a 9+5 pattern, another (*B*) shows a displacement of an outer doublet plus extra central tubules (8−1+4 pattern); the third cilium (*C*) shows a 9+4 pattern

Fig. 7. An example of axoneme fusion. Note the displacement of two outer doublets from one member of the pair

Table 1. Defects in ciliary axonemes of hpy/hpy mice[a]

	N	Dynein deficient	9+2	Central tubule anomalies	Displaced tubules	Double axonemes	Fused cilia
Normals	181	0	100	0	0	0	0
Mutants							
Trachea	251	36.4 (27.3–42.1)	65.3 (55.6–87.8)	23.5 (9.9–30.9)	8.0 (2.4–13.6)	3.2 (0–6.2)	0
Oviduct	243	39.8 (33.3–40.8)	94.5 (89.5–95.3)	4.3 (3.8–7.9)	1.2 (0.9–2.6)	0	0
Ependyma	235	32.0 (25.0–42.9)	88.0 (85.6–92.8)	6.0 (3.6–7.2)	0.9 (0–1.3)	0	5.1 (3.6–5.9)

[a] Values are expressed as percentages

for the trachea; in such axonemes the radial spokes frequently had a distorted appearance or were lacking. Tracheal cilia also differed from those of the oviduct and ependyma in that a low frequency (about 3%) possessed "double" axonemes. In these cases there appeared to have been a partial fusion of two axonemes with a displacement of up to three outer doublets of one member (Fig. 7). On the other hand, the presence of fused cilia (not illustrated) seemed confined to ependymal cells. No examples of the various abnormalities described above were encountered in any of the tissue samples from non-mutant animals. On the basis of the orientation of the central-pair tubules, the cilia of non-mutant epithelial cells were orientated in the same way. In the case of mutant cells, however, some cilia appeared as if they had been rotated through 45–90°; such abnormally oriented cilia were more frequently observed on cells from the ependyma.

Discussion

It is evident that the situation actually pertaining to ciliary axonemes of *hpy/hpy* mice is rather different from that anticipated on the basis of the previous findings for spermiogenetic cells (Bryan 1977 and 1981). Thus, ciliary axonemes exhibit a wide spectrum of anomalies, only certain of which have previously been recognized in their counterparts from spermiogenetic cells. Moreover, not all of them appeared to be present with the same frequency in all three epithelial types (Table 1). So while these investigations have provided answers to certain of the questions raised in the introduction they have, at the same time, raises other rather perplexing ones concerning the expression of the mutant gene. Hence, in order to attain a better understanding of the probable mode of action of the *hpy* gene it is necessary to account both for the instability of axonemes in spermiogenetic cells and the existence of other anomalies apparently confined to somatic cells. Because axonemes are assembled from a variety of structural components

numerous possibilities exist. In view of the normal-appearing initial assembly and growth of axonemes in early spermatids (c.f., Bryan 1983), it is evident that whatever defects are present would have to be rather subtle ones. The instability of axonemes in spermatids could stem from their being contructed from defective molecular components (tubulin subunits, microtubule-associated-proteins, radial spoke- or nexin link-proteins) which are not found in somatic cells. This would require the existence of different populations of such molecular components in somatic and germ cells. With respect to tubulin subunits, some support for such a view is that diverse cell types have now been shown to possess multiple forms of tubulins (c.f., Denoulet et al. 1982; Gozes and Barnstable 1982) and also that testis-specific mutant forms of tubulin have been identified in certain organisms (e.g., Kemphues et al. 1980). Additional support may be derived from the studies of Baccetti et al. (1979a) who have reported that in a certain male-sterile human patient sperm flagella axonemes lacked the central-pair tubules but ciliary axonemes were completely unaffected. With respect to other tubule-associated components little definitive information is at hand other than that in at least one case of Kartargener's syndrome in man, showing a deficiency of dynein arms, only one (the A_1 band) of the 4 dynein electrophoretic bands is present (Baccetti et al. 1979b). On the other hand, it is equally possible that the instability may arise as a consequence of defects in the makeup of the radial spokes and/or nexin links. In this event it would be expected that the resulting weakening of structural constraints normally provided by these structures would materially lessen, or abolish, the stability of the usual arrangement of the axonemal tubules regardless of whether or not the tubules were abnormally constituted. In some instances intact (9+2) and partial (9+0) axonemes with distorted spokes have been seen in early spermatids (c.f., Bryan 1983) but, owing to the scarcity of axonemes in the seminiferous epithelium of mutants, it is not clear whether or not they are representative. At the same time, ciliary axonemes with displaced tubules (central singlets, outer doublets) and distorted or absent radial spokes have been encountered in samples from each of the three epithelial types (Table 1); in all cases their microtubules contained typical numbers of protofilaments. In this connection it is of interest to note that similar gene-based anomalies have been encountered in axonemes of certain human patients (Sturgess et al. 1979). Thus, there is evidence both for the occurrence of mutant forms of various molecular components of axonemes and for their tissue-specific expression.

It is also conceivable that the primary action of the mutant gene results in a sequelae of events giving rise to the spectrum of abnormalities encountered and that site-specific modulations of this spectrum are responsible for the observed differences in expression. In other words, depending upon local conditions for the magnitude of their expression, certain defects may arise as direct consequences of the primary action of the mutant gene. In the absence of tissue-specific modulations the full spectrum of axonemal defects would be expressed to the same extent in all cell types. The existence of marked differences in the frequencies of certain abnormalities among

the three epithelial types (Table 1) might be taken as evidence favoring such a view. On this basis, and in view of the relatively rapid dismantling of putative axonemes in spermatids, it would appear reasonable to conclude that the condition characteristic of germ cells may represent the most extreme expression of the mutant gene. However, the observed variation in number of central tubules raises further complications regarding interpretation along these lines. Thus, there are two disparate sets of observations which must be dealt with: axonemes lacking central tubules, and axonemes possessing extra central tubules. It would appear as if mutually antagonistic processes are at work – one resulting in a reduction and the other an increase in their number. With respect to spermatids, it would appear reasonable, at this juncture, to assume that an absence of central tubules may be indicative of their preferential removal early in the dismantling process. At the same time, however, and because of their obvious stability, this would not appear to be a very satisfactory explanation of the occurrence (even though they are rare) of ciliary axonemes lacking central tubules. Instead, it would appear to be more reasonable to conclude that they may represent so-called *primary cilia* characteristic of embryonic cells (c.f., Sorokin 1968). In support of this are findings (Dalen 1981, and others) indicating that occasional cilia identifiable as primary cilia are present among the regular ones on mature cells. Normally, primary cilia are entirely replaced by typical ones by the time of birth but it appears that, in the adult, primary cilia may develop on cells destined to replace damaged or worn-out components of the epithelium (c.f., Dalen 1981). Although none were seen in the non-mutant samples in the present studies, the presence of primary cilia in the adult also affords a convenient explanation for the presence of cilary axonemes with displaced outer doublets. It has been demonstrated by means of serial sections (e.g., Dahl 1963; Flood and Totland 1977) that, at a short distance from the beginning of the ciliary shaft, one outer doublet becomes displaced centrally, transforming a $9+0$ into an $8+1$ pattern. Similar anomalies have also been reported in human patients envincing impaired mucobronchial clearance (Sturgess et al. 1980). Perhaps the increased incidence of such types of abnormalities in *hpy/hpy* mice betokens an increase in ciliogenetic- and cell replacement activities occasioned by an increased work-load stemming from the presence of significant numbers of dynein-deficient, immotile, cilia. If so, this would also provide a satisfactory explanation for why such abnormalities were not encountered in tissue samples from non-mutant littermates.

Accounting for the presence of cilia possessing extra central tubules is a much more difficult proposition. At first glance the observations would appear to imply anomalous microtubule-organizing activity on the part of the centrioles (basal bodies). One explanation would be to postulate that such aberrant cilia are the result of local (somatic) mutational events affecting individual centrioles (and their descendants). But this does not now seem very likely since, as pointed out recently (Dalen 1981), the view that centrioles contain their own genetic material has been effectively disputed. Currently, a more attractive idea is the occurrence of local errors during

the replicative process. Given the extensive proliferation of centrioles required to meet the needs of ciliated cell, it is entirely feasible that such activities would provide ample opportunity for occasional errors to occur. So it is conceivable that many cells might contain a small number of anomalous microtubule-organizing centers and, thereby, aberrant cilia. In this connection it may be of significance that similar ciliary anomalies have been reported to be present in samples of nasal and tracheal epithelium from various human patients suffering from gene-induced or certain other (non-genetically based) respiratory conditions (c.f., Bryan 1983). Most importantly, all of these conditions impair the effectiveness of the ciliated epithelium and presumably materially increase the work-load and wear and tear of the cells. Nevertheless, in the absence of clear indications of respiratory distress in *hpy/hpy* mice, this question must still remain an open one. If one accepts the assumptions implicit in the foregoing paragraphs it is possible to exclude from consideration as primary effects of the mutant gene such ciliary anomalies as variations in the number of central tubules and displaced outer doublets. In this event, the mechanism(s) resulting in the observed deficiency of dynein arms and, in spermatids, the unstable nature of the axonemes may be construed as the principal effects of the *hpy* gene. Then it would follow that, with the exception of axonemal instability in spermatids, the effects of the *hpy* mutation fall into line with those reported for similar mutations in man.

It is evident that a number of the questions raised by the findings cannot be fully resolved at this time. To do so will require additional detailed information concerning the molecular makeup of axonemes from spermiogenetic cells. In an attempt to provide such information various experimental approaches, some being addressed to the problem of axonemal stability, are currently under consideration. In the meantime, it is clear that some of the questions raised in the introduction have been answered. Thus, the identification of axonemes deficient in dynein arms in both somatic and germ-line cells of male mutants indicates that this effect of the mutant gene is not testis-limited. Similarly, finding this same defect in ciliary axonemes from mutant females signifies that the *hpy* gene is not sex-limited in its expression. Furthermore, in as much as all three epithelial types exhibit about the same level of dynein arm deficiencies, it would appear that a major effect of the mutant gene is expressed to essentially the same extent in each cell type.

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