the algae from *C. xamachana* and *C. frondosa* are *S. microadriaticum*, but that the others that we studied are distinct species.

As has been discussed (*6, 21–23*), there are several considerations that make the concept of a single species *S. microadriaticum* unreasonable. Although superficially these symbionts appear similar when freshly isolated from their hosts, they show stable and consistent differences in chemistry, physiology, behavior, and details of morphology when maintained in culture under uniform conditions. In addition, different hosts accept certain strains of *S. microadriaticum* as symbionts and repeatedly reject others. Altogether, the available evidence supports the concept that the binomial *S. microadriaticum* encompasses a large species complex. It appears that the systematic problems involving these algae are not unlike those pertaining to *Tetrahymena* (*24*), *Paramecium* (*25*), or *Crypthecodinium* (*26*), but unlike these organisms data on sexual recombination in *S. microadriaticum* are lacking. It is therefore not yet possible to test directly for speciation in these symbiotic algae in the context of the biological species concept, but the differences in chromosome organization of the various strains described here suggest a strong likelihood of severe cytogenetic problems if these strains could exchange genetic material in nature. We therefore suggest in the context of the evolutionary species concept (*27*), that the different strains are different species. Furthermore, we suggest that entirely different taxa of dinoflagellates, superficially resembling *S. microadriaticum*, may be involved in symbioses with marine invertebrates.

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17. The symbiotic algae used in this study were derived from *Cassiopeia xamachana* (and *C. frondosa*), the Jamaican sea anemone *Heteractis lucida*, the Californian sea anemone *Anthopleura elegantissima*, and the Hawaiian stony coral *Montipora verrucosa*. With the exception of the algae from *A. elegantissima*, all the cells analyzed were maintained under uniform conditions of culture (*6*). Cultured cells were harvested by centrifugation; algal cells from *A. elegantissima* were fixed immediately after isolation from the host. Fixation methods used included OsO_4 (20 mg/ml) in sea water or 50 m*M* cacodylate buffer (*p*H 8.1); 3 percent (by volume) glutaraldehyde in cacodylate buffer and then fixation in OsO_4; 3 percent (by volume) acrolein in 200 m*M* phosphate buffer (*p*H 8.1) and then fixation in OsO_4; or acrolein-glutaraldehyde fixation followed by OsO_4 fixation. Cells were stained en bloc with uranyl acetate (saturated in 70 percent ethanol by volume) and infiltrated and embedded in Spurr's resin [A. R. Spurr, *J. Ultrastruct. Res.* **26**, 31 (1969)]. Serial sections approximately 65 nm thick were prepared on an LKB Ultratome V and were viewed and photographed with a Philips-300 electron microscope. Morphometric analyses were conducted with a Zeiss MOP-3 using photographs printed at magnifications between ×20,000 and ×40,000 [R. Blank, E. Hauptmann, C. G. Arnold, *Planta* **150**, 236 (1980)].
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Chromosome-Sized DNA Molecules of *Plasmodium falciparum*

Abstract. *At least seven chromosome-sized DNA molecules (750 to 2000 kilobases in length and one fraction of undetermined molecular weight) from cultured clones and isolates of* Plasmodium falciparum *have been separated by pulsed-field gradient gel electrophoresis. Whereas asexual blood stages and sexual stages of the same line have identical molecular karyotypes, the length of chromosome-sized DNA molecules among different geographical isolates and several clones derived from a single patient is different. These length alterations of chromosomes are the result of DNA rearrangements that must occur unrelated to sexual differentiation.*

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The protozoan *Plasmodium falciparum*, the causative agent of one form of human malaria, is transmitted by an insect vector (*Anopheles* sp.) and undergoes extensive differentiation in its life cycle (*1*). The comparison of chromosome-sized DNA molecules (molecular karyotypes) of *P. falciparum* from different stages of its life cycle and from isolates from different geographical areas may give insight into the flexibility of the genome and the contribution of chromosomal recombination events to differentiation and the development of drug resistance.

Pulsed-field gradient (PFG) gel electrophoresis separates chromosome-sized DNA molecules up to at least 2000 kb in length (Figs. 1 to 3) (*2–6*). Degradation of DNA has been shown not to occur during the process, indicating that the separated molecules represent the DNA of the full-length chromosomes; they are therefore referred to as chromosome-sized DNA molecules (*2–6*). Molecules larger than 2000 kb are difficult to recover quantitatively. PFG electrophoresis relies on the variable capacity of DNA molecules of different lengths to reorient in electrical fields that are perpendicular to each other. Separation is dependent on the frequency of field switching, which critically affects the different molecular weight classes (*2–6*). Since the two electrical fields are applied alternatingly in the north-south and west-east direction, the molecules migrate at the diagonal of the electrical fields.

Gentle lysis of a mixture of asexual stages (ring forms, trophozoites, and schizonts) of *P. falciparum*, followed by separation of the chromosome-sized DNA molecules by PFG electrophoresis (*2–6*), allowed the visualization of at least seven chromosome-sized DNA molecules [Fig. 1a, samples 1, 2, and 3, six

bands and one fraction at the slot (*7, 8*)]. The approximate molecular weights of three fractions were determined by simultaneous analysis of *P. falciparum* with *Trypanosoma brucei* and yeast chromosome-sized DNA molecules and ligated λ multimers as markers. The smallest *P. falciparum* band migrated at 750 kb [above the 14th λ marker band, which is not shown (Figs. 1b and 3a)]. The largest resolvable *P. falciparum* chromosome-sized DNA molecules migrated with the 2000-kb *T. brucei* molecules (Figs. 1b and 3a). Furthermore, the four bands between 750 kb and 2000 kb migrate slower than the ninth chromosome-sized DNA molecule of yeast (at least 1000 kb). One fraction, which remained at the slot of the PFG gels, consisted of chromosome-sized DNA molecules of undetermined molecular weights as well as cellular debris. The varying intensities of the bands reflected variability in the number of copies of the chromosomes, as was also shown in the PFG analysis of *T. brucei* (*5*). This is visualized in the analysis of stabilate NF 7/8, which was derived from isolate NF 7/7 and lost the second smallest chromosome from the karyotype; therefore, there may only be a single copy of this chromosome [Fig. 1, sample 4, indicated with solid triangle (*8*)].

The genome complexity of *P. falciparum* is only inaccurately known and measures between 1×10^7 and 2×10^7 base pairs (bp) (*9, 10*). The six bands we observed, which varied in length from 750 to 2000 kb and of which at least three were double bands, therefore represent at least 50 percent (10^7 bp) of the genome. The remaining DNA migrated at the slot of the PFG gels. The presence of the double bands, and at least one molecule of undetermined molecular weight, indicates that the chromosome number must be more than seven and is likely to be at least ten. The same number of chromosome-sized DNA molecules was detected in other isolates and clones of *P. falciparum*. Since large circular molecules do not migrate, we did not expect to recover mitochondrial DNA (*2–6*).

The parasite, which is haploid in the mammalian host, undergoes sexual differentiation resulting in gametocytes and, in the mosquito, the formation of micro- and macrogametes (*1*). To establish the presence of sex-specific chromosomes and to determine whether chromosomal recombination contributes to differentiation, we compared gametocytes (Fig. 1a, sample 3), macrogametes (Fig. 1a, sample 2), and a mixture of asexual stages (Fig. 1a, sample 1). The

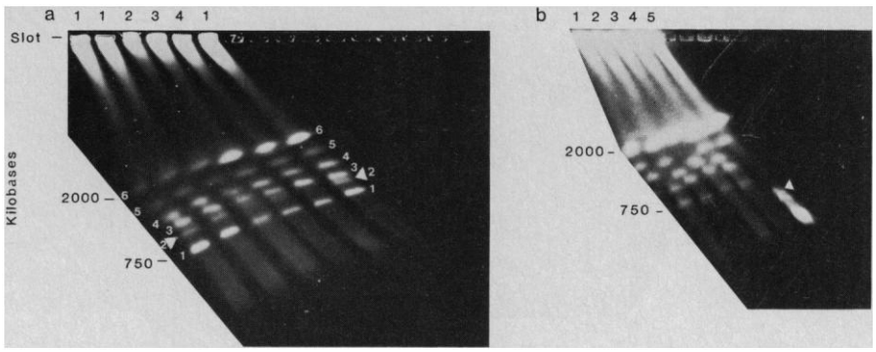

Fig. 1. (a) Separation of chromosome-sized DNA molecules of sexual and asexual stages of *P. falciparum* strains NF 7/7 and NF 7/8 (*8, 17*). The PFG electrophoresis was performed for 36 hours at 15°C, with pulse frequency of 70 seconds and with a field of 15 volts/centimeter in the north-south dimension and a field of 5 volts/centimeter in the west-east direction. The gel was stained with ethidium bromide after electrophoresis. The separation in a discrete molecular weight class depends on the pulse frequency and the length of time of the PFG electrophoresis; therefore, six bands are visible in Fig. 1a and five in Fig. 1b. The decrease in intensity of the largest molecules (2000 kb) between samples 1, on the left- and right-hand sides, is the result of mechanical shear of the larger molecules in this region of the agarose gel. Samples 1 contain the asexual blood stages of NF 7/7; samples 2 and 3 contain NF 7/7 macrogametes and gametocytes, respectively; and sample 4 contains the asexual stages of NF 7/8. (b) Ethidium bromide–stained PFG gel of asexual stages of clones obtained from a Thai isolate, T9, which were analyzed by Thaithong *et al.* (*7*). These clones vary in their isoenzyme patterns and resistance to chloroquine and pyrimethamine. The parasites were isolated (*16*) and treated at a pulse frequency of 80 seconds for 18 hours; lane 1, clone 94; lane 2, clone 96; lane 3, clone 102; lane 4, clone 101; lane 5, *T. brucei* stock 427 variant 118a (*4*).

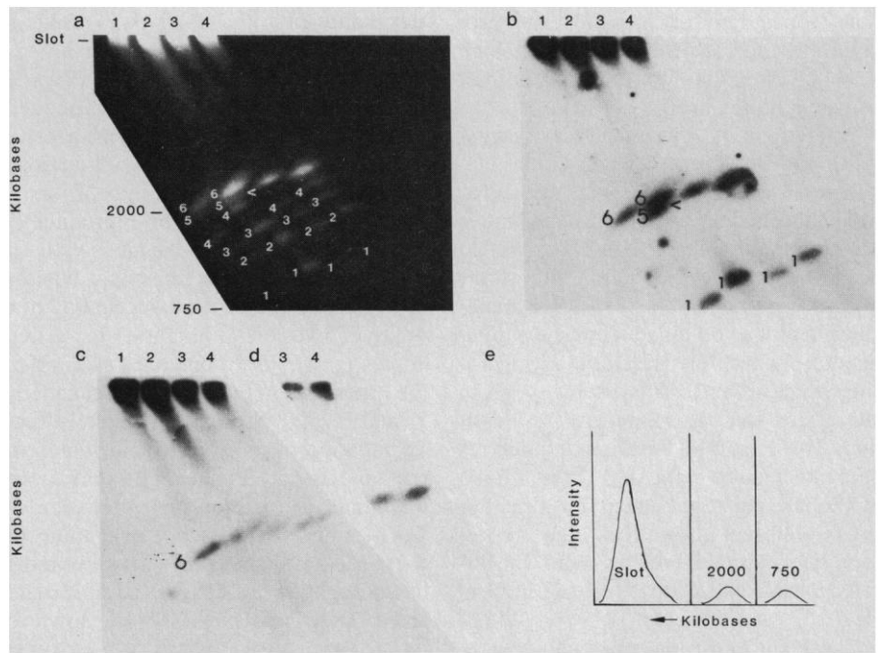

Fig. 2. (a) Ethidium bromide–stained PFG agarose gel of the isolates Brazil (lane 1); NF 54 (lane 2); NF 114 (lane 3); and NF 7/8 (lane 4) [see (*8*) for isolate and stabilate descriptions). Conditions of sample preparation were the same as for the samples in Fig. 1a and the electrophoresis procedure was as indicated for Fig. 1b. (b) Hybridization of the chromosome separation gel shown in (a) with ribosomal repeats (*12*). We have used a mixture of the Hind-III fragments of the clones pPF RIB 1 and pPF RIB 2 containing part of the 18*S*, the complete 5.8*S*, and part of the 28*S* ribosomal RNA genes. Washes after hybridization were performed at a stringency of 65°C and three times standard saline citrate (SSC). (c) Hybridization of the chromosome separation gel in (a) with the genomic clone pPF 11-1 specific for a blood stage antigen (*13*). The coding sequence of pPF 11-1 has a 29-bp repeat. The hybridization signal in the 2000-kb fraction increases with the use of lower pulse frequencies, which results in an equal distribution of the signals in the slot and the 2000-kb materials (d). This is probably due to mechanical shear and, therefore, loss of signal in the larger chromosome-sized DNA molecules at the higher pulse frequencies. (d) Hybridization of isolates NF 114 (lane 3) and NF 7/8 (lane 4) treated with a 50-second pulse frequency as in (c). Washes after hybridization were performed at a stringency of 65°C and one times SSC. (e) Densitometric scan of lane 1 in (b). The relative hybridization intensity of the three bands is 7:1:1.

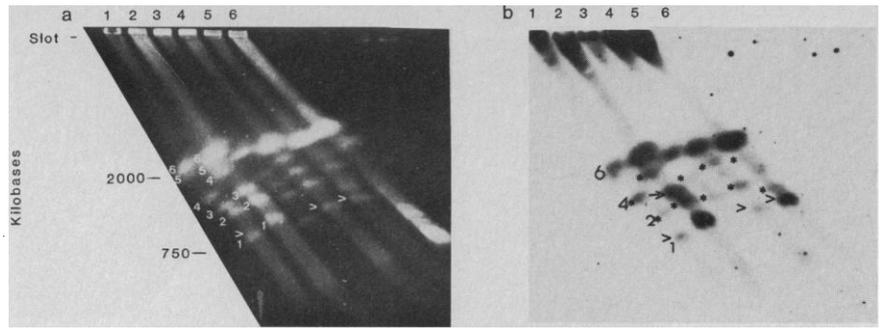

Fig. 3. (a) Ethidium bromide–stained PFG gel of the isolates; lane 1, Brazil; lane 2, NF 54; lane 3, NF 114; lane 4, NF 7/8; lane 5, NF 51; lane 6, *T. brucei* stock 427 variant 118a (*4, 13*). Conditions of sample preparation were the same as for the samples in Fig. 1a and the electrophoresis procedure was as indicated in Fig. 1b. (b) Hybridization of the PFG gel in (a) with a repetitive probe (λ pF rep. 20) obtained from *P. falciparum* (*7*). Washes after hybridization were performed at 65°C and three times SSC. Bands 2 and 4 in lanes 1 to 5 are indicated with asterisks, and band 3 in lane 2 is indicated with an arrow.

PFG analysis could detect no alterations in the molecular karyotypes between the sexual and asexual stages. We have, therefore, no evidence for extensive chromosomal rearrangements or the presence of sex-specific chromosomes that could contribute to the differentiation process. Other groups have reported that cloned haploid parasites can produce all stages of the life cycle, which also indicates that sex-specific chromosomes may be absent (*1, 11*).

Alterations in the molecular karyotypes can be detected when clones obtained from one patient [Fig. 1b (*7*)] or isolates from different geographical areas are compared (Figs. 2a and 3a). We do not know whether the length alterations of the chromosome-sized DNA molecules in clones obtained from the patient result from multiple infections with parasites that differ in karyotype or from alterations that arose during the infection. These length alterations, which reflect molecular weight shifts of hundreds of kilobase pairs, must be independent of sexual differentiation. They may, therefore, be random events or occur specifically in nonsexual stages of the life cycle.

The changes in the molecular karyotypes are not the result of preparation artifacts or modifications at the DNA level, such as DNA glycosylation or structural changes (that is, folding of the molecule). This is clear because:

1) The separation pattern of isolates is highly reproducible. Six different preparations of isolate NF 7/7 gave identical results in more than 30 experiments. Also, the separation patterns are independent of the differentiation state of the cell, as shown by the comparison of sexual and asexual stages.

2) When a ribosomal repeat probe is used (*12*), a specific hybridization pattern results. In isolate NF 54 the ribosomal repeats are found in four different fractions, whereas the same repeat is found in only three bands in the other isolates (Fig. 2, a and b, band 5, lane 2, indicated with arrowhead; Fig. 3a, band 5).

3) When a probe specific for the blood stage antigen pPF 11-1 (*13*) is used, a specific hybridization pattern results in two chromosomal fractions (Fig. 2, c and d). Another blood stage antigen encoded by probe pPF 2L (*14*) only hybridizes to the DNA at the slot. The hybridizations with probes pPF 11-1 and pPF 2L show the banding patterns to be reproducible and specific. Also, the quantitation of eight to nine different ribosomal repeats in *P. falciparum* (*12*) and our finding of a relative hybridization ratio of seven copies in the slot to one copy in each of the other bands (Fig. 2, a and b, bands 1, 5, and 6; Fig. 2e) make it unlikely that degradation of molecules contributes to the banding pattern. From these data we infer that degradation or aggregation of DNA molecules does not contribute to these separation patterns. Thus, we most likely separate full-length chromosome-sized DNA molecules, as was shown to be the case in the PFG analysis of yeast and *T. brucei* (*2–6*).

4) The alterations in the length of the DNA molecules most likely result from chromosome rearrangement events. This can be seen from the hybridization of the separated DNA molecules with a repetitive probe [λpF rep. 20; (*9*)]. This repeat hybridizes to most chromosome-sized DNA molecules. However, a comparison of bands of approximately the same length in different isolates shows variability in the hybridization intensities in lanes with identical DNA concentrations (Fig. 3, a and b, lanes 1, 4, and 5, bands indicated with arrowheads). In addition, a comparison of bands within one lane (for instance, Fig. 3b, bands 2 and 4, indicated with asterisks) shows intensity variations that do not result from DNA concentration differences. Also, band 3 in lane 2 of Fig. 3b hybridizes at the same intensity as band 2 in lane 2, whereas it is not detectable in most other lanes. These variations can only result from rearrangements at the DNA level. There could be many small deletions that alter the location of a repetitive element, or the variations could result from the presence of transposable elements. The length alterations of the chromosome-sized DNA molecules, the variable location of the ribosomal repeats, and the variable intensities of hybridization with the repeat probe, however, indicate that chromosome rearrangement events may give rise to the completely different molecular karyotypes in the various clones and isolates.

The chromosome rearrangement events in *P. falciparum* and those detected in clones of other protozoa such as *T. brucei* (*5, 15*) and *Leishmania tropica* (*16*) suggest that such rearrangements may be an intrinsic feature of protozoa. In *T. brucei* they may contribute to the regulation of differential gene expression (*5, 15*). It is not clear as yet whether this could also hold for differential gene expression in *P. falciparum*. The accumulation of mutations resulting from chromosome rearrangement events should be hazardous to the parasite. This may explain why continuous syringe passage, which does not provide the selection for mosquito transmission of *Plasmodium*, always results in lines that lose both the capacity to be transmitted by the mosquito and to undergo gametocytogenesis. The isolation of a spontaneous mutant in culture (NF 7/8; Fig. 1a) that had lost the capacity to produce gametocytes concomittant with changes in one of the small chromosomes may be indicative of this.

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8. NF 7 was isolated from a Ghanian returning to the Netherlands in December 1977. The parasites were introduced into continuous culture in January 1978 and kept for more than 4 years. NF 7/7 was derived from a frozen stabilate two weeks after initiation of the original culture. NF 7/8 was obtained from NF 7/7 and preserved at a time when its ability to produce gametocytes had been irreversibly lost (most likely a clonal selection). All stabilates of NF 7 are sensitive to chloroquine. NF 51 (a gift from D. Walliker) is

an FVO strain and is R3-chloroquine resistant; no gametocytes are produced in culture. NF 54 is an Amsterdam airport strain, isolated in August 1979; it is a gametocyte producer (used routinely in Nymegen and elsewhere for mosquito infection) and is chloroquine-sensitive. NF 114 was isolated in July 1984 from a Dutch patient returning from West Java, Indonesia; it produces gametocytes and is chloroquine-resistant. Brazil strain isolate IMTM 22 (a gift from R. S. Nussenzweig) is a gametocyte producer and is chloroquine-sensitive.
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18. *Plasmodium falciparum* asexual and sexual stages were produced in 10-ml suspension culture systems. Medium change was automated and occurred twice daily (*19*).
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Picosecond Time–Resolved Resonance Raman Studies of Hemoglobin: Implications for Reactivity

Abstract. *Picosecond time–resolved Raman spectra of hemoglobin generated with blue pulses (20 to 30 picoseconds) that were resonant with the Soret band and of sufficient intensity to completely photodissociate the starting liganded sample are reported. For both R- and T-state liganded hemoglobins, the peak frequencies in the spectrum of the deoxy transient were the same at approximately 25 picoseconds as those observed at 10 nanoseconds subsequent to photodissociation. In particular, the large R-T differences in the frequency of the stretching mode for the iron-proximal histidine bond ($\nu_{Fe\text{-}His}$) detected in previously reported nanosecond-resolved spectra were also evident in the picosecond-resolved spectra. The implications of this finding with respect to the distribution of strain energy in the liganded protein and the origin of the time course for geminate recombination are discussed. On the basis of these results, a microscopic model is proposed in which delocalization of strain energy is strongly coupled to the coordinate of the iron. The model is used to explain the origin of the R-T differences in the rates of ligand dissociation.*

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Although efforts to determine the functional and the structural properties of proteins have met with great success, a clear picture of how energy is stored within a protein and utilized for function is still not available. As a case in point, the static structure and functional properties of hemoglobin (Hb) are exceedingly well characterized (*1, 2*), yet even for this well-studied system a detailed microscopic account of the relation between structure and function is lacking. Part of the problem is that, although high-resolution x-ray crystallographic techniques provide detailed information about equilibrium structures, they cannot be readily used to probe the transient structures that link the initial and final functional states of a protein.

The equilibrium structures of ligand-free and ligand-bound Hb are well characterized (*1, 3*). In going from the ligand-free to ligand-bound protein, the local tertiary structure about the binding site responds to the change in both the quaternary structure (low-affinity T state → high-affinity R state) and the state of ligation at the heme (deoxy → liganded). To fully explore how protein structure controls the ligand-binding properties of the heme, it is necessary to determine how the local tertiary structure responds to functionally important perturbations (change in quaternary structure, ligand dissociation) and to establish correlations between specific structural features associated with the tertiary structures and parameters of ligand binding. One approach has been to study the ligand dissociation by rapidly photodissociating the ligand-bound protein and monitoring the subsequent dynamics of ligand and protein. Initial microsecond studies (*4*) focused on the bimolecular rebinding process, in which a ligand from the solvent rebinds to a heme surrounded by a protein that has partially relaxed from its initial structure. More recently, nanosecond (*5–7*) and picosecond (*8*) studies revealed much faster geminate (cage) rebinding processes. Transient Raman (*9–11*) and absorption (*7, 12*) studies have also probed the structural relaxation occurring on nanosecond and longer time scales. However, even the nanosecond Raman measurements (*13–16*) do not bridge the temporal and structural gap to the initial well-characterized structure of the liganded protein. We report here high-quality picosecond transient Raman spectra of photodissociated liganded Hb generated with blue, picosecond pulses of sufficient intensity to photolyze a sizable fraction of the starting material. The structural information in these spectra provides the link between the structure of the starting liganded Hb and the abundant functional and structural data obtained on the nanosecond and microsecond transients.

Single blue, picosecond pulses (435 nm, 10 mW, 10 Hz) were used to generate the low-frequency Raman spectra of the transient species that occurs within 20 to 30 psec after the photolysis of various R- and T-state liganded Hb's. Previous picosecond Raman investigations (*17*) in which low-intensity yellow pulses were used showed only that the transient form occurring within 30 psec of photolyzing COHbA (HbA, adult hemoglobin) had a high-frequency spectrum characteristic of a deoxy heme. Figure 1 shows a segment of the low-frequency portion of the picosecond Raman spectra from several different photodissociated COHb's. In each instance the frequencies of the spectral peaks at ~25 psec are identical to those of the corresponding deoxy transient at 10 nsec after photolysis. Substantial photolysis of the illuminated sample was achieved, as evidenced by the near-absence of the