± 50°. The rotation between the two tetramers A and B is, however, not defined by the dimensional features of the diffraction patterns, and it is, therefore, not possible to verify this hypothesis in detail.

The differences between the structures of the 3' and 5' isomers indicate clearly that the position of the phosphate group plays an important role in determining the structure of the linear aggregates. This is presumably because of the ability of the phosphate group to form hydrogen bonds with atoms on neighboring molecules, thus adding to the stability of particular configurations, as well as being due to the electrostatic repulsion of the charge on the phosphates. The further differences between the two preparations of the 3' isomer indicate that the structures can also vary according to the nature of the environment of the aggregate.

The large difference in optical rotation of gels of the 3' and 5' isomers is also consistent with the X-ray results. The 5' isomer forms a regular helix and would be expected to give a large rotation in the helical form,[9] whereas the 3' isomer consists of pairs of planar tetramers stacked on top of each other and would be expected to have a considerably different helix contribution to the optical rotation. The differences in optical rotation between preparations I and III of the 3'-GMP are not unexpected in light of the observed differences in the diffraction patterns.

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STREPTOMYCIN AS A MUTAGEN FOR NONCHROMOSOMAL GENES

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Stable hereditary determinants segregating in a non-Mendelian manner were first described in 1908 by Correns.[1] In the following decades, some hundred or more well-established examples of non-Mendelian heredity were reported,[2, 3] but few attempts have been made to integrate them into a general theory of genetics. The principal difficulty blocking a systematic study has been the rarity of their occurrence and their failure to respond to mutagenic agents.

The chance isolation, some years ago, of a mutant of the alga *Chlamydomonas reinhardi*, exhibiting nonchromosomal inheritance of streptomycin resistance (*sr*-500),[4] provided a new material with which to reinvestigate the role and origin

of nonchromosomal genes.[5, 6]   This paper reports studies in which streptomycin has been found to act as a mutagen of nonchromosomal genes, and thereby as a source of novel genetic material for analysis.

Materials and Methods.—The organism, *Chlamydomonas reinhardi*, employed in these studies has been previously described, as have the culture conditions and crossing techniques.[4, 6, 12] All studies of mutation to streptomycin resistance were carried out with strain *21 gr*, which is mating-type + (*mt*+).   The streptomycin-induced mutants of y_1 which gave rise to the auxotrophs reported in this paper, came from a *mt*+ strain carrying *sr-500* as well as the chromosomal gene markers *act-r* (actidione-resistant) and *ms-r* (methionine sulfoximine-resistant) previously described.   M and Ac media correspond to media I and II respectively described previously.[12] M hi P medium is M plus the high phosphate of Ac, but no acetate; and Ac lo P medium contains the acetate concentration of Ac but the low phosphate of M.   Recrystallized streptomycin, the gift of Merck & Co., was used in all induction and assay experiments.   Streptomycin-resistant mutants were classified for phenotypic resistance level by spotting loopfuls of suspensions of log phase cells onto agar with various concentrations of streptomycin and scoring for growth.   Under the test conditions, sensitive cells do not grow on drug-containing agar, and *sr-100*'s do not grow on M plus 300 or 500 μg of streptomycin per ml, used to distinguish between *sr-100* and *sr-500* mutants.   In each experiment, the inoculating culture was grown from a fresh single colony isolate. Cultures were assayed for numbers of *sr* mutants either by spread or pour-plate techniques; the two methods gave comparable results.   In the fluctuation analysis experiments, the complete tubes were assayed.   When necessary, more than one plate was employed per tube to avoid plate suppression.

Results.—*Phenotypic distinction between chromosomal and nonchromosomal mutations:*   In the course of this investigation, some 10,000 streptomycin-resistant clones of independent origin were classified for resistance level and found to fall into two classes: most of them were resistant to 100 μg of streptomycin per ml on agar, as shown by the ability of every cell to form a colony; and a few per cent were resistant to 500 μg per ml.   Crosses of 84 of these strains, chosen at random, revealed a strict correlation between resistance level and pattern of inheritance.   In crosses to a streptomycin-sensitive strain, 57 of the mutants, resistant to 100 μg per ml but killed by 300 μg per ml, each showed 2 : 2 segregation in tetrad analysis of the progeny, indicating the chromosomal basis of the mutation.   These mutants are referred to as the *sr-100* class.   On the other hand, 27 mutants, resistant to 500 μg per ml, each showed the same nonchromosomal pattern of inheritance as that initially described[4] and are referred to as the *sr-500* mutants.   Resistance was transmitted uniformly to all progeny, when carried by the *mt*+ parent, and exhibited no segregation in the F_1 or in subsequent backcross generations.   Since the resistance level was found to correlate closely with the pattern of inheritance, the resistant colonies recovered were classified by phenotype and only a sampling was further tested by crosses.

Mutant frequencies: Initial observations of the frequencies of *sr-100* and *sr-500* mutants in streptomycin-sensitive (wild type) populations (Table 1) revealed behavior on the part of *sr-100* mutants similar to that described for bacterial system[7] but two anomalies in the appearance of *sr-500* mutants.

Considering the *sr-100* class first, the frequency of mutants was about the same regardless of the plating medium used for selection of resistant mutants so long as the streptomycin concentration did not exceed 100 μg per ml (Table 1).   The recovery of a few *sr-100* mutants at higher streptomycin concentrations is consistent with their phenotypic behavior.   Some physiological variation in resistance level occurs, allowing occasional survival at higher drug concentrations.   An unexpected

TABLE 1
MUTANT FREQUENCIES OF sr-100 AND sr-500 UNDER VARIOUS CONDITIONS

Culture Media		Mean frequency of sr-100	Mean frequency of sr-500
Inoculum	Assay plates		
M	M + 100 μg S/ml	6×10^{-6}	3.7×10^{-9} (2 only)
Ac	Ac + 100 μg S/ml	5.5×10^{-6}	3.4×10^{-7}
Ac	Ac + 200 μg S/ml	1.5×10^{-6}	1.7×10^{-8}
Ac lo P	Ac lo P + 100 μg S/ml	1.3×10^{-6}	4.6×10^{-8}
M hi P	M hi P + 100 μg S/ml	1.8×10^{-6}	6.6×10^{-9} (2 only)

finding was the suppression of the appearance of sr-100 mutants by crowding. The apparent frequency dropped about 10-fold when 5–10 $\times$ 10^6 cells were plated. In all experiments with sr-100 reported here, unless specifically noted, no more than 2 $\times$ 10^6 cells were spread per plate. Suppression was found not to affect the appearance of sr-500 mutants when less than 10^7 cells were plated.

Considering now the sr-500 mutants, one anomaly was noted in comparing the frequency of mutants arising on M and on Ac media containing streptomycin (Table 1). About 100 times as many mutants appeared on Ac + 100 μg/ml as on M + 100 μg/ml. Ac differs from M in containing sodium acetate and a 30-fold higher concentration of phosphate, but the pH of the two media is the same. In tests of the independent effects of acetate and of phosphate, it appeared that acetate contributed more to the increase in mutant yield than did phosphate. Other media, supplemented with yeast extract and amino acids, did not alter the mutant yield.

In an attempt to account for the difference in mutant yield, comparative studies were carried out of the behavior of wild-type cells on four media. Sensitive cells plated on Ac + 100 μg/ml undergo 2–3 residual doublings before they die, whereas on M plus streptomycin, there is at most one doubling. There is also a slight effect of the liquid medium in which cells are grown as inocula for the assay plates, with Ac liquid conferring some protection against streptomycin. These differences, while of interest, clearly do not account for the 100-fold difference in yield of sr-500 mutants on M and Ac drug-agar plates. Also, these media do not significantly affect the average frequency of mutations to sr-100.

It seemed possible that survival time itself might be a relevant parameter in accounting for the difference in yield of sr-500 mutants on the various media. To test this possibility, populations of sensitive cells were plated on streptomycin-containing M and Ac media, washed off at intervals, and replated at dilution on M lacking streptomycin to assay for survivors. In 24 hr, survival was less than 1 per cent on M + S medium but 100 per cent on Ac + S, falling to 1 per cent only after 96 hours. Ac lo P + S was intermediate. These results indicate a strong correlation between survival time of cells in the presence of the drug and yield of sr-500 mutants. Such a result is suggestive of a chemically induced mutagenic process.

A second anomalous feature in the origin of sr-500 mutants, as shown in Table 1, is the decrease in yield of mutants on plates with 200 μg of streptomycin per ml compared to those with 100 μg/ml, despite the ability of the mutant clones, once isolated, to grow well with 500 μg per ml. This result too suggests that the process giving rise to sr-500 mutants differs from that of spontaneous mutation, in which the full capacity for resistance is established before contact with the drug. (Old strains of sr-500 mutants kept many years in the absence of the drug retain their full re-

sistance level and do not behave like new mutants when challenged with 500 μg of streptomycin per ml.)

In contrast to these results, the appearance of sr-100 mutants in the same experiments was fully consistent with their origin as pre-existing spontaneous mutations not induced or altered but merely selected by streptomycin.

Fluctuation analysis: The Luria-Delbruck fluctuation test[8] provides a critical method for distinguishing between spontaneous and induced mutation, based upon the difference in time of occurrence of mutations of the two types. Spontaneous mutations may occur at any time in the growth of a culture. In a series of small cultures, each started from a few cells, the number of mutants per tube will show a high variance if assayed at a suitable time with respect to the mutation rate. Induced mutations, however, will occur with a fixed probability and show a normal (in these experiments, Poisson) distribution with a low variance. This method, originally developed for the analysis of the origin of bacterial mutations, was applied to the origin of sr-100 and sr-500 mutations in *Chlamydomonas*.

Initially, fluctuation analysis experiments were carried out with cells grown on Ac liquid and plated out for assay on Ac + 100 μg of streptomycin per ml. Under these conditions, sr-100 mutants appeared with a variance from tube to tube (Table 2) significantly higher than that of the control set of tubes pooled before assay (Table 3). In the same experiment, the sr-500 mutants showed a very low variance, providing no evidence of their origin as spontaneous mutations accumulating in the tubes before assay.

TABLE 2
FLUCTUATION ANALYSIS

	Liquid cultures grown on Ac and plated on Ac + 100 μg streptomycin per ml		Liquid cultures grown on Ac and plated on M + 300 μg/ml		Liquid cultures grown on M and plated on M + 100 μg/ml
No. of tubes	30	37	37	34	30
Cells/tube					
initial	200	82.5	37	270	800
final	5.5×10^6	9.9×10^5	5×10^7	3.8×10^7	1.5×10^7
Mean No. mutants per tube sr-100	1.4	3.0	2.1	5.3	5.3
Variance	6.0	39.0	27.0	250.0	48.0
P	0.1	0.005	0.005	0.005	0.005
Mean no. mutants per tube sr-500	0.1	0	0.19	0.32	0.1
Variance	0.09	—	0.17	0.23	0.093
P	>0.9		0.75	0.2	>0.9
Mutation rate/cell/ division sr-100 (P_0 method)	1.1×10^{-6}	8.2×10^{-7}	4.5×10^{-9}	9.2×10^{-9}	3.6×10^{-8} (partial plate suppression)
Mutation rate/cell/ division sr-500 (P_0 method)	1.4×10^{-7}		2.4×10^{-9}	3.6×10^{-9}	4.6×10^{-9}

TABLE 3
VARIATION IN THE NUMBER OF MUTANTS IN ALIQUOTS SAMPLED FROM A SINGLE POOLED CULTURE

	Mean	Variance	X^2	P
Experiment 1	1.4	1.6	0.0133	>0.9
" 2	0.71	0.83	0.009	>0.9
" 3	0.55	0.28	0.095	>0.25
" 4	2.1	2.1	0	∞

In view of the likelihood that sr-500 mutants arose as plate mutants on Ac-streptomycin agar, it seemed worthwhile to examine the pattern of origin of these mutants on M-streptomycin agar, on which few if any plate mutants arise. In a fluctuation test assayed on M + 100 μg of streptomycin per ml, sr-500 mutants were found with a frequency comparable to that initially observed in this medium (Table 1), but the variance was low. In contrast, sr-100 mutants, although showing some suppression as a result of plating at 7.6×10^4 cells, nonetheless, exhibited a high variance characteristic of pre-existing mutants, as in previous experiments.

Since the number of sr-100 mutants appearing in these experiments could be altered either by suppression or by increasing the streptomycin concentration, it was possible to find conditions under which both sr-100 and sr-500 mutants were appearing with approximately the same frequency. This was achieved by growing up the tube cultures in Ac liquid, and then plating to assay for mutants on M plus 300 μg of streptomycin per ml. (Growth in M liquid in a parallel experiment gave no mutants at all when cells were plated on M plus 300 μg of streptomycin per ml, but apparently the growth in Ac liquid provided some protection against rapid killing by streptomycin.) Both sr-100 and sr-500 mutants were recovered, with similar mutation rates, as shown in Table 2, but again the variance of sr-100 mutants was very high, and that of sr-500 mutants was low and did not differ significantly from the mean.

These results demonstrate that sr-500 mutants, in contrast to sr-100's, do not accumulate in liquid cultures as pre-existent mutants before contact with streptomycin. One may still ask whether there is something inhibitory in liquid culture and whether spontaneous mutations to sr-500 might occur on agar. To test this possibility, the Newcombe respreading method,[9] which is essentially a qualitative equivalent of fluctuation analysis, performed on agar, was applied.

Newcombe respreading analysis: In this method, cells are spread on a series of plates and allowed to grow. At intervals, a suitable number of plates are removed and divided into two sets. One set is respread to break up any developing mutant clones, and the other set is left unspread, both then being layered with streptomycin-agar to kill the wild-type background, leaving the streptomycin-resistant cells to develop into colonies. The increment in number of sr colonies found on the spread plates over those on the unspread plates provides a direct demonstration of the appearance of sr clones on plates before contact with streptomycin.

The origin of sr-500 mutants was studied by plating cells on Ac agar, and layering with Ac + 300 μg of reptomycin per ml after respreading. Under these conditions, only sr-500 mutants appeared on the plates. In the first experiment, over a 72-hr period, with a 260-fold increase in cell population, there was no differential increase in number of mutants on the respread plates. A reconstruction experiment was carried out simultaneously with an initial mixture of wild-type and sr-500 cells in the ratio of $10^6:2$. The expected increase in number of sr mutants occurred on the respread plates, demonstrating that pre-existing sr-500 mutants cells can multiply and form colonies under the conditions of the experiment. Similar results were found in the second experiment, which, together with the first, corroborate the previous findings of the fluctuation test, that sr-500 mutant clones do not develop before contact with streptomycin.

Streptomycin treatment of cells in liquid culture: In view of the evidence that

streptomycin was mutagenic, it seemed possible that higher yields of mutants might be obtained by suitable manipulation. Accordingly, an extensive series of experiments was carried out, including short treatments in liquid culture with various concentrations of the drug, exposure of cells to the drug under nongrowing or slow-growing conditions, use of a variety of different media, etc. Although it was possible to vary the survival and to decrease the yield of *sr-500* mutants greatly by different treatments, the yield of *sr-500* mutants was not consistently increased over that on Ac agar + 100 μg of streptomycin per ml.

"Phenocopies": A by-product of the liquid culture studies was the discovery of transient streptomycin resistance, displayed by *sr* "phenocopies." Sensitive cells were grown for two hr in liquid M medium with 500 μg of streptomycin per ml and then plated on M agar. Survival was about 20 per cent but colonies appeared slowly and erratically. Beginning at 6 days after plating, whole microcolonies (about 10^4 cells) were picked and tested for streptomycin resistance. All colonies were sensitive except the most delayed group, appearing after 10 days. Of the microcolonies tested at 12 days, 55 per cent grew on M agar with 300 μg of streptomycin per ml and half of these grew for another subculture on streptomycin, a total of 14–20 doublings in the presence of the drug. This behavior contrasts sharply with that of sensitive cells which die after one doubling at most.

Streptomycin-induced auxotrophic mutants: Forty streptomycin-induced y_1 mutants[10] have been re-examined and all but four of them found to exhibit some growth factor requirement. The predominant mutant type, which has been isolated from 11 of the strains, is acetate-requiring, that is, nonphotosynthetic. These mutants are green in light, grow well on minimal medium supplemented with acetate, and are stable on subculture. In liquid culture tests of four strains, growth was proportional to the acetate concentration in the medium.

One strain, S-12, unlike the others, grows as well with yeast extract as with acetate. The growth factor in yeast extract which spares the acetate requirement has not yet been identified, but in its absence growth is proportional to the acetate concentration of the medium, as in the case of the other strains. S-12 and two other acetate-requiring strains, S-1 and S-10, all mt+, have been crossed to a tester stock (mt−), and the progeny has been scored for acetate requirement and for the segregating chromosomal markers act-r/act-s and ms-r/ms-s, as well as for *sr-500* and y_1. The results demonstrate that the acetate-requirement is transmitted to all progeny without segregating, in the same manner as *sr-500*. Y_1 segregated independently, as in previous crosses.

Thus, it appears that a new class of auxotrophic mutants was induced by streptomycin in the same treatment as that which led to mutation of y_1. They behave, however, like the *sr-500*, *sr-1500*, *sd* class of mutants, in inheritance,[6] transmitting their acetate-requirement to all progeny with no segregation.

Discussion.—This paper reports the finding that streptomycin is mutagenic for certain classes of genetic determinants: those which are nonchromosomal in location. In a previous study,[10] the mutation of y_1 under the influence of streptomycin was described. In the present study, mutations from streptomycin sensitivity (wild type) to resistance were shown to be of two types: chromosomal gene mutations (*sr-100*) arising at a rate of 0.8–1.1 × 10^{-6} mutations per cell per generation in the absence of streptomycin; and nonchromosomal mutations (*sr-500*), identical

with the one originally described,[4] induced by streptomycin, as shown by fluctuation analysis[8] and by the respreading technique.[9]

In addition to sr-500 and y_1, a new class of streptomycin-induced mutants has been found among the y_1 mutants previously isolated after streptomycin treatment. Those analyzed thus far are acetate-requiring (nonphotosynthetic), but other phenotypes are also found. Three strains (mt^+) have been crossed with wild-type (mt^-), and each showed the same nonchromosomal pattern of inheritance in the F_1 as sr-500: that is, uniform transmission of the acetate requirement from the mt^+ parent to all progeny with no segregation occurring. Thus, nonchromosomal genetic determinants affecting such diverse biochemical capabilities as chlorophyll biosynthesis, streptomycin resistance, and prototrophy have all shown susceptibility to streptomycin as a mutagen. These results clearly indicate that the mutagenic action is directed against the determinants themselves irrespective of their functional activity.

The origin of sr-500 mutations: In studying the origin of mutations to sr-500, the problem of distinguishing between induced mutation and selection was a difficult one. This problem had previously undergone extensive analysis with bacterial systems,[7] in the course of which the methods of fluctuation analysis,[8] respreading,[9] and indirect selection[13, 14] were developed. All these methods demonstrated unequivocally the occurrence of spontaneous mutations to drug resistance, independent of the presence of the drug. In a quantitative study, it was concluded that all streptomycin-resistant mutants were of spontaneous origin.[15] In our analysis of the origin of sr-500 mutants of *Chlamydomonas*, we followed the bacterial methodology. The methods of indirect selection could not readily be applied for technical reasons, but both fluctuation analysis and the respreading method of Newcombe were applicable. Chromosomal gene mutations to sr-100 showed the same evidence of pre-existence before contact with streptomycin as had been described for the bacterial systems. However, neither method revealed the presence of any clones of sr-500 mutants pre-existing before contact with streptomycin, either in liquid culture or on agar, although sr-500 mutants did arise as plate mutants, on the streptomycin-agar assay plates.

A crucial difference in experimental design between the *Chlamydomonas* experiments and the bacterial ones, concerns the assay plates, on which the number of mutant cells present in the fluctuation analysis tubes are counted. For accurate counts, it is important that no residual growth occur on the assay plates, so that no new plate mutants can arise. This result could be achieved with *Chlamydomonas* by growing liquid cultures in M and plating on M + 300 μg of streptomycin per ml. Under these conditions, no sr-500 mutants were found. If cells were grown on Ac liquid, however, sufficient survival resulted on M + 300 μg plates to permit the appearance of sr-500 mutants at a frequency of about 10^{-9}. Plating on Ac + 100 μg led to frequencies at least 100-fold higher. In fluctuation tests, the mutants exhibited a Poisson distribution, on both plating media, with no evidence of mutant clone formation in tubes before plating.

Because of the possibility that liquid culture conditions might somehow interfere with the origin of growth of sr-500 mutants, analogous experiments were carried out on agar with respreading. Here, too, no evidence was found for the pre-existence of sr-500 mutants before contact with the drug. In view of these results with *Chlamy-*

domonas, it is instructive to reconsider the bacterial studies, in which experiments were designed specifically to avoid the appearance of plate mutants. Thereby they excluded any bacterial mutants analogous to the *sr-500* class, which in our experiments appear *only* as plate mutants.

The mutagenic action of streptomycin: Studies of the origin of *sr-500* mutations have shown that the mutagenic process is not a simple one. Conditions favoring the appearance of these mutants include the presence of acetate, the use of toxic amounts of streptomycin, and a long lag before the appearance of mutant clones. Similar conditions were required for the occurrence of streptomycin-induced mutations of y_1,[10] although there the parental strain was carrying *sr-500* and required ten times as much streptomycin for mutagenesis as did the wild-type strain used in the present studies.

The absence of nonchromosomal mutants following short treatments of sensitive cells with streptomycin in liquid culture has thus far blocked a systematic study of the biochemistry of the mutagenic process. The production by a short exposure to the drug, of phenocopies, transiently resistant to streptomycin, raises the possibility that phenocopy production may be a step on the pathway to mutation. If so, biochemical studies become more feasible, since the yield of phenocopies is some 5% of the treated population. What biochemical changes would one expect?

Ever since the first demonstration that streptomycin combines readily with nucleic acid,[16] attempts to account for the biological potency of this drug have frequently invoked reactions with DNA or RNA. In a general sense, it would not be surprising that such a reactive compound would exhibit mutagenic activity. There is evidence, however, that it is not an effective mutagen for chromosomal genes, either in *Chlamydomonas* or in bacteria.[17] Both this fact and the peculiarly slow and traumatic conditions under which nonchromosomal mutations appear suggest that the mutagenic action which we have studied does not involve DNA directly.

RNA merits consideration as an autonomous carrier of genetic information in normal cells, for a number of reasons. The occurrence of RNA viruses in plant, animal, and bacterial cells demonstrates that RNA *can* carry genetic information. Furthermore, the replication of viral RNA requires the existence of an enzymatic mechanism. A recent report of RNA with stable, heritable transforming ability for penicillin resistance, isolated from *B. subtilis*[18] represents the first direct evidence of a nonviral genetic RNA. If the genetic determinants mutated by streptomycin are RNA, then studies of RNA biosynthesis and degradation under the conditions giving the highest yields of phenocopies, may be revealing. Effects of streptomycin on membrane permeability,[19] and on the structure and function of ribosomes,[20–22] are also under investigation.[23, 24]

Summary.—The mutagenicity of streptomycin for nonchromosomal determinants has been studied. The drug induces mutation of a nonchromosomal gene from streptomycin-sensitivity to resistance, as shown by fluctuation analysis and respreading experiments. Auxotrophic mutants were induced by growth of streptomycin-resistant cells with toxic concentrations of the drug. Three of these strains, which are acetate-requiring, have been crossed and found to show the same nonchromosomal pattern of inheritance as the streptomycin-resistant strains. The streptonycin-induced loss of chlorophyll-synthesizing ability, also under the control of a nonchromosomal determinant,[11] was reported elsewhere.[10] Thus, the mu-

tagenic effect of streptomycin is nonspecific, being directed toward a class of molecules carrying genetic information rather than toward a particular determinant.

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GENETICS OF HUMAN CELL LINES, IV. DNA-MEDIATED HERITABLE TRANSFORMATION OF A BIOCHEMICAL TRAIT

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The phenomenon of DNA-mediated genetic transformation,[1] first recorded for Pneumococcus,[2] and later extended to several other bacterial species and to infectious viral DNA was only recently demonstrated to occur in mammalian cells.[3, 4] That the phenomenon may be more generalized in mammalian systems is suggested by the related observations: the uptake of nucleic acids by mammalian cells,[5-15] and the infectivity of naked nucleic acids isolated from mammalian viruses.[16-18]

Numerous attempts have been made to demonstrate genetic transformation in mammalian cells,* but the systems employed apparently were not sufficiently selective. In the majority of cases the experiments were performed partially or entirely