number; however, for seven pairs of fields A + C = 12 and for two pairs A + C = 14 and thus for these fields duplication of a number does not occur if the sum of A and C is coded. It is not possible to code ten digits without using the sum of A + B or B + C.

The first field found, the first example in Table 1 (which shows the 22 new fields) (*2*) was put into practice and has presented no problems in use.

A photograph of the card designed is shown in Fig. 1, punched to indicate the system used. The numbers 1, 8, and 3 are encoded by shallow punching; 6, 10 (or 0), and 7, by deep punching; and 5, 2, and 4, by intermediate punching. Shallow punching of 1 and 8 encodes 9; shallow punching of 8 and 3 gives 11 and ambiguity is avoided. Shallow punching of 3 and 1 could give an ambiguous 4 but this is avoided by the convention that the sum of A and C should not be used for coding a number.

Recently the question arose of coding any letter of the alphabet by using only one field. The letters have been coded by punching the number which corresponds to their position in the alphabet; thus A is coded by punching the number 1, and P is coded by punching the number 16. The letters U and V are both coded by punching the number 21, and the letters X and Z, which occur rarely, are coded by not punching the card. The numbers from 11 to 20, inclusive, may be punched in more than one way; this ambiguity was overcome by arbitrarily deciding how these numbers should be coded. For convenience a reference card was made on which the alphabet was punched (see Fig. 2).

The average number of needle passes required to separate one letter from the rest is 3.71, while for the 7-4-2-1-NZ system (*3*) the average is 4.54. The alphabetical coding has been applied to a rapidly growing deck of cards (5000 at present) which are frequently searched with no deterioration of the card stock.

It is possible to make a field on a double-row card, using only two edge holes, by using combinations of shallow, intermediate, and deep punching, but these holes could not be numbered, and while more fields could be put onto a card more time would be spent searching, since almost every hole of the four would be punched in some manner.

ALAN G. NEWCOMBE
Newcombe Associates, 25 Dundana Avenue, Dundas, Ontario, Canada

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4 March 1963

Replicating Form of a Single-Stranded DNA Virus: Isolation and Properties

Abstract. *The replicating form of single-stranded DNA virus has been isolated in pure form by chromatography on columns of methylated albumin. Its buoyant density in CsCl and "melting temperature" are characteristic of a double stranded DNA structure containing 43 percent guanine-cytosine. The nearest neighbors to uridylate were compared in the RNA synthesized when replicating-form DNA and mature single-stranded DNA were employed as templates in an in vitro system. The mature DNA component of the replicating duplex does not serve as the sole source of complementary RNA. The results agree best with the assumption that both strands of the replicating form function as templates. It is important to note that this is contrary to the situation found in the intact cell where only one of the two strands appears to be transcribed into message.*

Sinsheimer (*1*) has shown that the DNA virus, ΦX174, contains only one of the two possible strands. Further, on injection into cells of *Escherichia coli*, the other complement is synthesized (*2*). The resulting duplex constitutes what has been called the "replicating form" or RF.

This situation provides an obvious opportunity for an informative experimental analysis of the genetic-transcription mechanism. However, the fact that the mature virus provides one of the two strands can be fully exploited only if the replicating form can be purified as a source of both complements. It is the purpose of the present paper to show how this can be achieved. The purified RF-DNA has been used to gain a more direct knowledge of its physico-chemical properties. In addition, its behavior as a template for in vivo transcription into RNA has been compared with the product synthesized on the single-stranded DNA isolated from virus particles.

The replicating form was detected (*2*) in the DNA of infected cells as a unique component in a cesium chloride density gradient (*3*). It was clear, however, that neither the resolution nor the capacity of this method would suffice for the convenient preparation of the RF-DNA in adequate quantities. Experience in other laboratories (*4, 5*) as well as in our own (*6*) with methylated-albumin columns (*7*) led us to believe that chromatographic separation of the RF should be possible.

To help identify the position of the RF-DNA on the column, the DNA of the virus used for infection was labeled with phosphorus-32. In addition, the infection and incubation was carried out in the presence of chloramphenicol which inhibits (*2*) the formation of mature single strands. Figure 1 shows the result of chromatographing the total DNA isolated and purified from the *E. coli*–ΦX174 complex after 1 hour of incubation. Included in the material put on the column was tritium-labeled single-stranded ΦX174–DNA which is eluted at a unique position in the gradi-

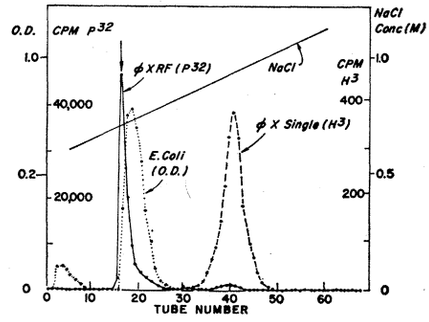

Fig. 1. Separation of ΦX174 replicating form from ΦX174-single-stranded DNA on a column of methylated albumin on kieselguhr (MAK). The *E. coli* C and ΦX174 were from the virus stock of Dr. I. Tessman. Its DNA was prepared and labeled with P^{32} or H^3-thymidine (*16*). The columns were prepared (*4*) with well-methylated (*16*) albumin. Our standard column is one-half the size used routinely by Mandell and Hershey (*4*). Other sizes used are denoted by ½ X, 10 X, and so forth. Log-phase (1×10^9 per ml) cells (100 ml) were infected with P^{32}-labeled ΦX174 (multiplicity of 10 in the presence of 50 μg/ml of chloramphenicol). After 1 hour of incubation at 37°C the infected complexes were harvested and the total DNA was isolated (*18*). Two mg of the DNA were loaded on a 2X MAK column along with H^3-labeled ΦX174-DNA. The indicated linear NaCl gradient was applied and fractions were collected for measurement of optical density at 260 mμ and for the determination by liquid-scintillation counting on Millipore membranes of the radioactivity in the acid-insoluble portion (see *19*).

ent. Well-separated from the H^3-component is a sharp peak of P^{32}-labeled DNA—that is eluted at a NaCl concentration somewhat lower than that which suffices for the host DNA—identifiable by the optical-density (O.D.) profile. It would appear that on injection, the P^{32}-labeled DNA is converted into something which chromatographs in a very different position from that which characterizes the incoming strand. The fact that the P^{32}-DNA is eluted before the tritiated single-stranded DNA is to be expected if the P^{32}-strand has been made into a double-stranded structure by synthesis of the complement. This pattern is in complete accord with the deductions derived from the analysis in CsCl gradients (*2*).

Examination of the P^{32}-peak of Fig. 1 in an analytical ultracentrifuge yields expected evidence of heterogeneity as a broad peak encompassing the densities corresponding to the DNA of ΦX174–RF-DNA (ρ = 1.707 g/ml) and *E. coli* (ρ = 1.714 g/ml). Furthermore, the peak contains infectious DNA which yields ΦX174 particles when tested with protoplasts from *E. coli* C (*8*).

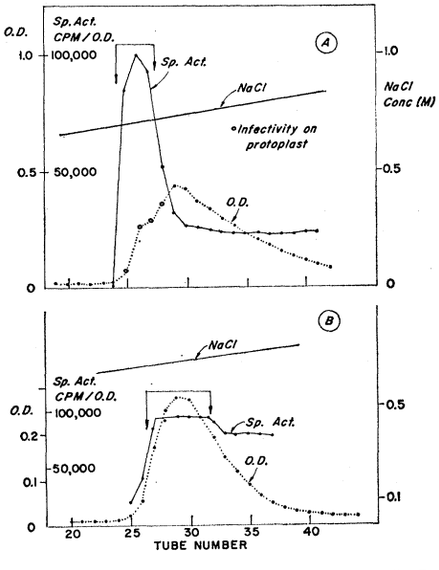

Fig. 2. Shallow-gradient separation of *E. coli* and RF-DNA. *A*, One liter of an *E. coli* C log-phase culture (1×10^9 cells per ml) was infected with ΦX174 (multiplicity of 10) in 10^{-3} *M* phosphate *SC X D* medium (*16*) containing 10 μc of P^{32} per ml and supplemented with 10 μg/ml each of deoxyadenosine, deoxycytidine, deoxyguanosine, and thymidine. Incubation was at 37°C with aeration. Ten minutes after infection 50 μg/ml of chloramphenicol was added, and 50 min later the cells were harvested and the DNA was isolated from the complex and chromatographed on a 5X MAK column. Measurements of O.D.^{260} and radioactivity were made as in Fig. 1. The fractions denoted by an open circle were the only ones that contained infectious DNA. *B*, The fractions enclosed within the brackets of Fig. 2*A* were pooled and rechromatographed on a ½X MAK column.

The relative positions of the P^{32}-DNA and *E. coli*-DNA suggest that separation of the two can be achieved by using shallower gradients of sodium chloride and by repeated chromatography. This was done in our experiments.

To prepare adequate amounts of the RF the chromatographic procedure was adjusted to employ columns ten times the size of those in the experiment of Fig. 1. As an aid in identifying the RF-DNA component, P^{32}-labeling was used during the infection. Because of the much smaller starting amount the specific activity of the RF-DNA would be expected to be considerably higher than that of the host DNA. This procedure also provides a material in which both strands of the RF complex would be labeled. To avoid the synthesis of mature strands the experiment was carried out in the presence of chloramphenicol.

The chromatographic pattern of the total DNA purified from the infected complex 60 minutes after infection is shown in Fig. 2*A*. The shallow sodium chloride gradient effects a better separation between the RF-DNA and the host DNA. A shoulder in the O.D. profile, characterized by a high specific activity, is clearly discernible at the NaCl molarity corresponding to the P^{32}-labeled material of Fig. 1. Samples were taken from various parts of the column to examine them for infectivity in protoplasts. Infectious DNA was found only in the samples denoted by a circle indicating that this type of DNA is confined to the region corresponding to high specific activity.

The fractions enclosed within the brackets of Fig. 2*A* were pooled and rechromatographed on a smaller column (1X) that yielded the pattern observed in Fig. 2*B*. The constant specific activity across the peak region suggests that comparative purity has been achieved. A third chromatography of the pooled material included within the brackets of Fig. 2*B* yielded essentially the same pattern. The purity of the preparation was further examined by density-gradient centrifugation (Fig. 3*B*) in CsCl by using nitrogen-15–DNA from *Pseudomonas aeruginosa* as a density marker (ρ = 1.746 g/ml). Figure 3*B* shows that the RF preparation yields a single symmetrical peak (ρ = 1.707 g/ml) with no evidence of distortion resulting from heterogeneity. Figure 3*A* represents the profile obtained when single-stranded ΦX-DNA is added to the RF-DNA preparation to the extent of 15 percent contamination. Its presence is readily detected at its characteristic density position (ρ = 1.725 g/ml). Calculation (*9*) of the percentage (in moles) of guanine-cytosine (GC) of the RF-DNA from its position in the density gradient yields a figure of 43.6 percent GC. This is in complete agreement with that expected from a duplex composed of a single strand from the

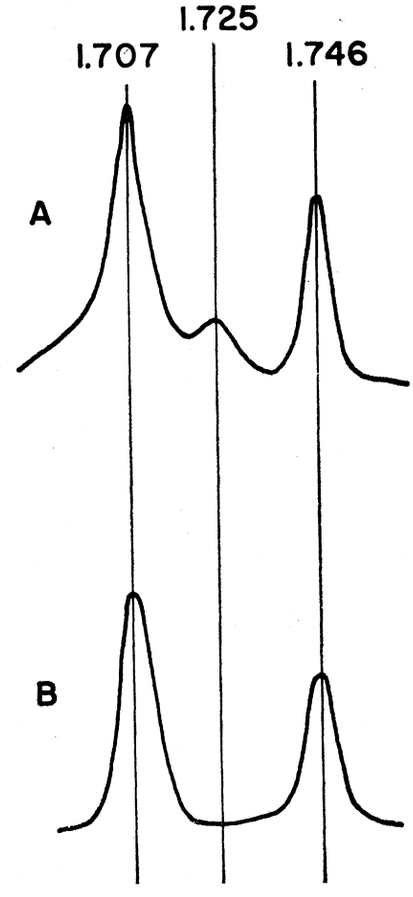

Fig. 3. CsCl density gradient centrifugation of purified ΦX174–RF-DNA. *A*, Seven micrograms of ΦX174–RF-DNA (from pooled bracketed fractions of Fig. 2*B*) and 2 μg of ΦX174 single DNA were centrifuged in CsCl. N^{15}-DNA from *Ps. aeruginosa* was used as a reference DNA (ρ = 1.746 g/ml). The input ρ of the CsCl was 1.710 g/ml. *B*, Seven micrograms of ΦX174–RF-DNA and N^{15}-DNA from *Ps. aeruginosa* were centrifuged in CsCl. The picture was taken after 20 hours centrifugation at 44,770 rev/min, and was traced by a Joyce densitometer. The density and percentage GC in DNA were calculated according to Sueoka (*9*).

mature virus and its complement. The yield of purified RF-DNA was routinely 200 to 300 μg for each liter of infected cells processed.

It was of further interest to examine the hyperchromic properties of the replicating form. In addition to providing evidence relevant to its secondary structure it could also yield an independent estimation of the percentage of GC from the relation of Marmur and Doty (*10*). Figure 4 compares the melting curves of the RF-DNA, single-stranded DNA from ΦX174, and double-stranded DNA from *E. coli*. The melting curve of the RF-DNA is similar in its sharpness to that of double-stranded *E. coli* DNA and is quite unlike the gradual rise in O.D. exhibited by the single-stranded DNA derived from the mature particles of ΦX174. The midpoint of the RF-DNA melting curve is 86.5°C and appropriate calculations indicate a base composition of 42 percent GC, a value in good agreement with that deduced from the density-gradient centrifugation.

Examination of the cooling curve of Fig. 4 suggests that renaturation of RF-DNA occurs more readily than that of *E. coli* DNA. This may be due to the greater simplicity in sequence complexity of the viral as compared with *E. coli* DNA or to an as yet unresolved peculiarity in its structure preventing complete strand separation.

We finally come to the template characteristics of the RF in the in vitro system. Chamberlin and Berg (*11*) have already made an interesting and relevant observation. They had synthesized a double-stranded structure by using the single strand of ΦX174 as a template with the Kornberg DNA-polymerase reaction (*12*). The resulting product was used as a template for the DNA-dependent RNA polymerase. Analysis of the RNA synthesized showed that both strands of DNA were transcribed. It was conceivable that the naturally occurring duplex might contain a control mechanism limiting the transcription to one of the strands. It was of interest, therefore, to re-examine this question with the isolated RF-DNA as a template.

For comparative purposes, RF-DNA and single-stranded ΦX174-DNA were tested as templates in the present experiments. Experimental details and results are summarized in Table 1 in the form of a nearest-neighbor analysis. With the single-stranded ΦX-DNA as the template, the only prediction

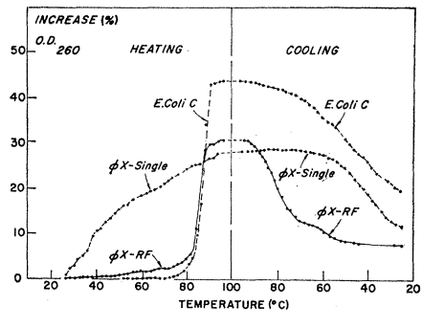

Fig. 4. Comparison of melting curves of purified RF-DNA, single-stranded ΦX174-DNA and native *E. coli*-DNA. A mixture containing 15 μg/ml of each of the DNA preparations was made in SSC buffer (*18*). Optical densities at 260 mμ at the ambient temperatures were measured in an "Opticon" spectrophotometer equipped with a heating unit. Temperature was increased at the rate of 1°C/2 min until the temperature reached 96°C. The cooling diagram was obtained during a schedule in which the decrease from 96°C to 60°C occurred over a 3-hour period.

possible is based on the assumption that it serves to direct the synthesis of an antiparallel complementary strand of RNA. The expected distribution of neighbors nearest to the uridylate are calculated from the data on ΦX174-DNA of Swartz *et al.* (*13*). The values found in the RNA product are in excellent agreement.

When the RF-DNA is used as the template the following three outcomes are possible. (i) Only the original strand is transcribed, in which case the distribution should be the same as recorded in the first item of Table 1. (ii) Only the complement of the mature strand is copied, leading to a prediction of nearest-neighbor distribution given in item 3 of Table 1; (iii) Both strands are transcribed, which predicts the results calculated in item 4 of Table 1. Comparison shows that the first prediction is easily distinguished from the others but that differentiating between items 3 and 4 is more difficult. The actual distribution in the product RNA when the RF-DNA served as the template (line 5) demonstrates clearly that the incoming strand is not the only one that serves as a template. The data appear to agree with the calculations which assume that both strands of the RF-DNA are transcribed. This is in accord with other investigations (*11*, *14*) of DNA transcriptions in cell-free systems. They leave open, however, the question of whether this situation obtains in vivo.

The availability of the RF-DNA in pure form permits one to go to the next stage of the investigation and inquire whether both strands serve as sources of genetic messages in the cell. This can be accomplished by hybridization tests (*15*) of RNA messages syn-

Table 1. Nearest neighbor analysis of RNA synthesized in vitro on single-strand and RF-DNA of ΦPX174. The DNA-dependent RNA polymerase was isolated from *E. coli* B (*11*). The specific activity of the purified enzyme was 6000 units (*11*) per mg protein, with calf-thymus DNA as a template. The reaction mixture (0.5 ml) contained 100 μg of template DNA, 20 μmole of tris buffer, *p*H 7.9, 0.5 μmole $MnCl_2$, 2 μmole of $MgCl_2$, 6 μmole of β-mercaptoethanol, 79 μg of enzyme, 100 mμmole ATP, CTP, GTP, and UTP^{32}, labeled in the nucleotide phosphorus. After incubation at 37°C for 90 min, the contents of the reaction mixtures were precipitated with 3 percent PCA and washed with 3 percent PCA five times. Four milligrams of *E. coli* bulk RNA was then added to each tube, hydrolyzed with alkali (0.3*N* NaOH) at 37°C for 15 hours. Chromatographic analysis of the 2′-3′-nucleotides was performed with a Dowex one-formate column (*19*). Thirteen millimicromoles of UMP^{32} were incorporated into RNA when single-strand DNA was used as a template. Twenty-one millimicromoles of UMP^{32} were incorporated when RF-DNA was the template. CpU, ApU, UpU, and GpU are the doublets C and U, A and U, U and U, and G and U, linked by phosphorus through a 3′,5′-carbon (*20*).

	Comparison of theoretical with experimental	Nearest neighbors to uridylate (percentage mole fractions)			
		CpU	ApU	UpU	GpU
	DNA template: ΦX*174-Single*				
(1)	Predicted* (one strand, mature strand as template)	22	30	29	19
(2)	Found (average of duplicates)	21.2 ± 0.2	29.4 ± 0.4	30.5 ± 0.3	18.9 ± 0.1
	DNA primer: ΦX*174-RF-DNA*				
(3)	Predicted† (one strand, complement of mature strand as template)	22	23	33	22
(4)	Predicted‡ (both strands, as templates)	21.4 to 22.5	25.8 to 27.7	31.4 to 34.0	18.2 to 19.6
(5)	Found (average of duplicates)	22.3 ± 0.2	24.3 ± 0.2	34.6 ± 0.3	18.8 ± 0.6

* Calculated from limited replication of ΦX174–DNA of Swartz *et al.* (*13*); it is assumed that the RNA synthesized is complementary and antiparallel. † Calculated from limited replication of ΦX174–DNA (*13*); and the data is converted into a complementary antiparallel template for the formation of an antiparallel RNA complementary to it. ‡ Calculated from the extensive replication of ΦX174–DNA in the experiments shown by Swartz in his table II (*13*). The number pairs represent the two extremes estimated from the nearest neighbors actually observed.

thesized in vivo with RF-DNA and the single-stranded DNA of the mature virus. Such experiments have been carried out and are described in detail elsewhere (*16*). The data demonstrate that in the intact cell only one strand is transcribed and that it corresponds to the complement of the one found in the mature virus particle (*17*).

M. HAYASHI
M. N. HAYASHI
S. SPIEGELMAN
Department of Microbiology, University of Illinois, Urbana

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20. Abbreviations: ATP, GTP, UTP, CTP, adenosine, guanosine, uridine, and cytidine triphosphates, respectively; UMP, uridine monophosphate; cpm, count/min; PCA, perchloric acid; SSC, 0.15*M* NaCl, .015 Na citrate.

7 May 1963

Cretaceous, Paleocene, and Pleistocene Sediments from the Indian Ocean

Abstract. *Two deep-sea cores containing Cretaceous, Paleocene, and Pleistocene sediments from an oceanic rise approximately 500 miles southeast of Cape Town contained well-preserved fossil foraminiferal ooze made up of about 97 percent planktonic forms, including species of* Guembelina *and* Hedbergella. *High percentages of particles less than 53 μ in diameter in the Cretaceous and Paleocene sediments indicate a deep-water open-ocean depositional environment. These sediments are the oldest recovered so far from the Indian Ocean.*

Pre-Pleistocene rocks were first dredged in the Indian Ocean on the west side of Providence Reef (9°25.8′S, 50°56.8′E). Wiseman (*1*) has described these Eocene and Oligocene basaltic agglomerates. Wiseman and Riedel (*2*) found Tertiary Radiolaria in two samples of sediments of 20°40′S, 85°29′E, and at 8°45′S, 64°52′E.

In recent years, 87 long, submarine cores from the Indian Ocean have been collected by Lamont Geological Observatory. Two sets of cores taken from near the top of a rise are described in this report. One long, piston core and its short, trigger-weight core contained sediments of Paleocene age; the other piston and trigger-weight cores contained Cretaceous deposits.

The piston-core sediments are white compacted calcilutite overlain by 7 to 8 cm of light beige, friable Pleistocene foraminiferal calcilutite mixed with older sediments. The trigger-weight cores contain rounded pebbles, granules, sand, compacted calcilutite, and friable manganese oxide nodules. The old sediments contained planktonic Foraminifera, calcareous and arenaceous benthonic Foraminifera, Ostracoda, Radiolaria, and coccolithophorid plates. Discoasters were not found. A few fish-teeth fragments and echinoid spines were the only megafossils found. *Inoceramus* prisms were observed only in the Cretaceous sediments. The fossils indicate an open-ocean environment.

The microfossils were identified under a binocular microscope after the sediment had been washed through a sieve which retains particles larger than 53 μ.

Core V16-55. Location; 40°14′S, 25°15′E. Core length, 179 cm; water depth, 2770 m.

Trigger weight core. Megascopic description: Several alter igneous rock fragments and compacted calcilutite and manganese oxide nodules.

Piston Core. Megascopic description: At 0 to 7 cm: Pleistocene, light tan, friable foraminiferal lutite. Contact blurred by burrowers.

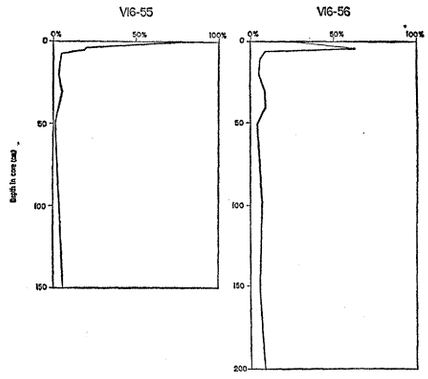

Fig. 1. Variation in percentage of coarse fraction (> 53 μ) in cores containing pre-Pleistocene sediments.

At 7 to 179 cm: white compacted Paleocene calcilutite.

At 100 to 179 cm: micronodules of manganese oxide present.

Calcium carbonate content: top, 90 percent; 100 cm, 89 percent.

Micropaleontological description: 0 to 1 centimeter: Pleistocene Foraminifera make up 98 percent of the total foraminiferal assemblage. Out of these, 2 percent are benthonic species. About 2 percent Paleocene Foraminifera were present at this level. In addition, echinoid spines, glauconite, and quartz grains were seen.

Between 4 and 6 cm in depth, the Pleistocene Foraminifera were still making up the bulk of the washed sample. The number of Paleocene Foraminifera increased, making up 5 percent of the faunal assemblage.

In the Paleocene section (7 to 179 cm), the following index species of planktonic Foraminifera were identified: *Globigerina triloculinoides, Globorotalia pseudobulloides, Globorotalia mokannai, Chiloguembelina victoriana, Chiloguembelina crinita, Globorotalia compressa, Globorotalia whitei, Globorotalia elongata* (*3*). Benthonic Foraminifera make up 3 to 4 percent of the washed foraminiferal fauna. In addition, Ostracoda, Coccoliths, and fish-teeth fragments were found.

Core V16-56. Location: 41°21′S, 26°38′E. Core length, 240 cm; water depth, 2950 m.

Trigger-weight core. Megascopic description: several friable manganese oxide nodules were recovered.

Piston Core. Megascopic description: At 0 to 8 cm: Pleistocene, light tan, friable foraminiferal lutite mixed with Cretaceous faunal remains; base indefinite, blurred by burrowers. At 8 to 240 cm: white compacted Cretaceous foraminiferal calcilutite. The calcium carbonate content of a sample taken at 100 cm depth is 89 percent.

Micropaleontological identification: At 0 to 1 cm: Pleistocene Foraminifera make