A VIRAL DNA-PROTEIN COMPLEX IN SV40 INFECTED CELLS

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ABBREVIATIONS

The abbreviations used in this thesis are those approved by the editorial board of the Biochemical Journal (Biochem. J., 116 (1970) 1). The following abbreviations are also used;-

ME	Mouse Embryo
	Mercaptoethanol (page 64)
PBS	Phosphate Buffered Saline
PDI	Propidium Di-iodide
PFU	Plaque Forming Units
SDS	Sodium Dodecyl Sulphate
SV40	Simian Virus 40

CONTENTS

Page

INTRODUCTION

SV40 virus - Introduction.

The widespread use of tissue cultures derived from monkey kidneys gave rise, in the 1950s, to a number of reports of indiginous monkey viruses recovered from uninoculated cultures (Hsiung & Gaylord,1961). Frequently, uninoculated cultures were observed to degenerate spontaneously to give a distinctive cytopathology. During production and testing of poliomyelitis vaccine, numerous filterable, transferable, cytopathogenic agents other than poliovirus were encountered (Sweet & Hillman, 1960).

The Simian Vacuolating Virus (SV40) was first isolated from uninoculated kidney cultures from rhesus, cynomolgus and patas monkeys (Sweet & Hillman, 1960 (b); Hsiung & Gaylord, 1961) and was found to be capable of inducing tumours in the Syrian hamster (Eddy et al, 1962) and multimamate rat (Robson et al, 1962).

SV40 and the closely related polyoma virus are both members of the Papovavirus group (Melnick, 1962). Where appropriate, SV40 and polyoma virus will be discussed together.

Physical and chemical properties of SV40 virus.

The virion, i.e. the extracellular mature virus, consists of deoxyribonucleic acid (DNA) contained within a protein shell, the capsid. Electron microscopy indicates that both polyoma virus (Mattern et al, 1967) and SV40 (Anderer et al, 1967) have icosahedral symmetry and contain 72 sub-units or capsomeres (Klug,1965). Each virion may contain 60 hexamers (each containing 6 identical polypeptide chains) and 12 pentamers (each containing 5 identical polypeptide chains) (Caspar & Klug,1962). Table A lists the physical and chemical properties of both polyoma and SV40 viruses.

During the course of purification of both polyoma and SV40 virions, 'empty' particles are always observed (Crawford, Crawford & Watson, 1962; Black, Crawford & Crawford, 1964). 'Empty'particles are readily distinguished from 'full' particles on the bases of their lower buoyant density, lower sedimentation coefficient and on electron microscopic analysis using negative staining (Black, Crawford & Crawford, 1964; Anderer et al, 1967).

Viral particles with a lower buoyant density than normal and which contain smaller DNA molecules than normal are produced in SV40 (Uchida et al, 1968;Yoshiike, 1968; Uchida & Watanabe, 1968) and polyoma (Thorne et al, 1968; Blackstein et al, 1969) infected cells at high input

TABLE A. Physical and chemical properties of SV40 and polyoma viruses. (from Green, 1970)

	SV40	Polyoma
Particle weight (daltons)	17×10^6	25×10^6
Diameter of virion (nm)	40	40-45
Molecular weight of DNA (daltons)	3×10^6 *	$2.9\text{-}3.4 \times 10^6$
%weight of protein	88	88
% weight of DNA	12	12
Buoyant density of virion (g/ml)	1.34	1.34
Sedimentation coefficient of virion (svedbergs)	219+	238x

* from Sebring et al, (1971)

+ from Koch et al (1967)

x from Crawford & Crawford (1963)

multiplicities (i.e. high input virus /cell ratios). These "defective" particles may be deletion mutants. Some defective SV40 particles can induce the synthesis of virus specific proteins (Uchida et al, 1968) and others are as oncogenic in newborn hamsters as fully infectious virus (Yoshiike, 1968).

Virus structural proteins.

In addition to its DNA, the SV40 virion contains one major polypeptide of molecular weight 43,000 (VP1) which comprises 70% of the total protein in the virion and five minor polypeptides of molecular weights 32,000 (VP2; 9%), 23,000 (VP3; 10%), 14,000 (VP4; 6%), 12,500 (VP5; 4%), and 11,000 (VP6; 3%). The molecular ratios of the polypeptides in the virion are 6.0: 1.0: 1.5 : 1.5 : 1.1: 1.0 (Estes et al, 1971). Polyoma virus has a very similar capsid protein complement (Roblin, Harle & Dulbecco, 1971). In the case of SV40 virus, the number of polypeptide chains of VP1 and VP2 has been estimated as 320 and 54 respectively, close to the sub-unit values as postulated by the hypothesis that there are 72 sub-units in the virion. (Caspar & Klug, 1962). This data is consistent with the idea that VP1 might be the polypeptide for building the hexamers and VP2 the polypeptide for building the pentamers.

When the virion is degraded in alkaline buffer, and analysed by velocity centrifugation through a sucrose

gradient, the polypeptides VP1 and VP2 remained at the top of the gradient whereas the three smallest polypeptides (VP4, VP5, and VP6) sedimented as a complex with the viral DNA (Estes et al, 1971). Similar results have been obtained by Friedmann (1972) for polyoma virus.

The small DNA genomes of polyoma (Follet & Crawford, 1968) and SV40 (Sebring et al, 1971) are estimated to contain sufficient genetic information to code for about 200,000 daltons of protein if only one strand of the viral DNA is transcribed in vivo (see later). The total molecular weight of polypeptide in the virion varies from 140,000 to 180,000 and would therefore saturate a very large fraction of the viral genome. There are also many proteins the synthesis of which are induced by the virus and which are not considered to be part of the virus particle;these include the tumour, transplantation and surface antigens (Black et al , 1963; Habel & Eddy, 1963; Tevethia et al, 1965; Tevethia et al, 1968) and certain enzymes (Kit et al, 1966; Kit et al, 1967). In order to overcome the difficulty that virus induced proteins account for more than the coding potential of the viral genome, one must postulate that some components are not virus-coded but derive instead from the host cell.

The three smallest structural proteins of polyoma virus, which are associated with the viral DNA, contain no tryptophan (Roblin, Harle & Dulbecco, 1971) and this fact suggests that these components might be host cell histones. To date, there are no known histones which contain tryptophan (Bonner et al, 1968) and the molecular weights and lysine to valine ratios of these three structural components (Roblin, Harle & Dulbecco, 1971) are consistent with the available data on histones (Butler et al, 1968). Frearson and Crawford (1972) and Hirt (1972) have recently shown that the three smallest structural proteins of both polyoma and SV40 have the same mobility on gels and similar fingerprints as extracts of basic nuclear proteins obtained from uninfected host cells.

Viral DNA.

The major proportion of the DNA extracted from purified polyoma or SV40 virus is in the form of a closed circular duplex (component 1) (Dulbecco & Vogt, 1963; Weil & Vinograd, 1963; Crawford & Black, 1964). It was later shown that these molecules are supercoiled, or twisted upon themselves (Vinograd, Lebowitz, Radloff, Watson & Laipis, 1965; Bauer & Vinograd, 1968).

Supercoiling is due to a deficiency, rather than an excess, of Watson - Crick turns in the DNA double helix, as shown by experiments with ethidium bromide (3,8-diamino-6-phenyl-5-ethylphenanthridium bromide) (Crawford & Waring, 1967). Ethidium bromide is a phenanthridine dye which forms a reversible complex with double stranded DNA. Dye molecules intercalate between the base pairs of the DNA, causing a local unwinding of the double helix. Addition of increasing amounts of ethidium bromide to supercoiled DNA results in a gradual removal of supercoiling until a non-supercoiled conformation is obtained. This would only be possible if supercoiling was due to a deficiency of turns in the double helix. The deficiency of right-handed (Watson-Crick) turns in the double helix can be made up for by the molecule adopting a conformation with right-handed twists, or supercoils. Further addition of ethidium bromide to supercoiled DNA results in the re-appearance of supercoiling, this time in a left-handed sense. Supercoiling may also be removed by partial denaturation, either by heating to 40 C in formaldehyde (Crawford & Black, 1964) or at pH 11.6 (Vinograd et al, 1965). The supercoiled form of SV40 viral DNA has a sedimentation coefficient of 21S at pH 8.0.

The component 2 form of polyoma or SV40 DNA consists of open circular duplex molecules and results from the introduction of a single-stranded scission of one phosphodiester bond in the component 1 form of the viral DNA (Vinograd et al, 1965).This form of viral DNA has a sedimentation coefficient of 16S at pH8.0.

Component 3 is mainly degraded cellular DNA, which has been encapsulated instead of viral DNA in virus particles and has a linear double-stranded structure (Michel, Hirt & Weil, 1967; Winocour, 1967; Levine & Teresky, 1970). Virions containing such cellular DNA are known as pseudovirions and, in the case of SV40, appear to occur to a greater extent when the virus is grown in primary culture cells, rather than in cell lines (Levine & Teresky, 1970).

The restricted uptake of ethidium bromide, or its analogue, propidium di-iodide (PDI) (Hudson et al, 1969), by closed circular double stranded DNA molecules forms the basis of a convenient method for their detection and isolation (Radloff et al, 1967). When ethidium bromide binds to DNA, it causes an unwinding of the helix about the duplex axis. However, closed circular double stranded DNA molecules possess no site within the molecule about which such an unwinding process could occur, and therefore resist the uptake of dye in large amounts. Closed circular DNA duplexes bind fewer molecules of dye/nucleotide than

open duplexes and, as a consequence of this, have a higher buoyant density and can therefore be readily separated from the latter by equilibrium density gradient centrifugation.

Infection of cells with SV40.

SV40 can interact with cultured cells in two different ways. On the one hand, there is a productive, or lytic, response in which the great majority of the cells yield progeny virus and die. On the other hand, there is an incomplete infection in which little or no virus is produced and the cells survive (abortive or non-permissive infection). Some of these surviving cells assume a new set of stable properties which closely resemble the properties of cells derived from tumours and continuously express certain viral functions. These cells are said to be transformed. Which consequence virus infection produces is solely determined by the species of the host cell. Non-permissive cells presumably lack some function(s), of unknown nature, essential for virus replication. Table B lists the cells that are commonly used in studies of lytic infection and transformation by

SV40 and polyoma.

TABLE 2

	Lytic infection	Abortive infection
SV40	Monkey	Rat, Mouse, Human
Polyoma	Mouse	Hamster

(A) Infection of permissive cells.

The replication of SV40 virus is studied in established monkey kidney cell lines e.g. BSC-1, CV-1 and in primary African Green Monkey Kidney (AGMK) cells.

The following sections will deal with the events which occur after infection of permissive cells with SV40.virus. Events are termed either "early" or "late" dependent on whether or not they precede the onset of viral DNA replication.

(1) Adsorption, penetration and uncoating of the virus.

Experiments in which monkey cells were infected with radioactive SV40 virus (Barbanti-Brodano, Swetly and Koprowski, 1970) have revealed that the virus initially

becomes associated with the cell membrane. Adsorption may be localised at specific receptor sites on the cell surface. After transport through the cytoplasm, the virus particle enters the cell nucleus where the viral DNA is uncoated and where the viral replication events ultimately take place. The total amount of free parental SV40 DNA in the nucleus progressively decreases (Barbanti-Brodano et al, 1970). Hirai & Defendi (1972) have recently demonstrated that a small portion of the infecting viral DNA becomes associated with host cell DNA by alkali-stable linkages. The significance of integration during the early phases of the replicative cycle of the virus cannot be clearly assessed at the present time.

Tai et al (1972) have demonstrated that 7-12% of the closed circular DNA molecules from SV40 virus grown at high multiplicity of infection contain a region in which a new base sequence has replaced the normal one. These new sequences are detected as non-homology regions in heteroduplexes. Lavi & Winocour (1972) have also shown that the type of SV40 DNA synthesised in BSC-1 cells depends upon the conditions under which the cells are infected. Viral DNA molecules containing sequences homologous to cell DNA are produced in cells infected with serially passaged plaque-purified virus,

or with non plaque-purified virus at high multiplicity of infection, whereas viral DNA which hybridises detectably to host DNA is not produced in cells infected with plaque-purified virus or in cells infected with non plaque-purified virus at low multiplicity of infection. Host cell DNA might be incorporated into SV40 DNA by a mechanism similar to production of lambda transducing phage. During the lytic infection, SV40 viral DNA would become integrated at one or more sites into host cell DNA (as occurs in the transformation process (Sambrook et al, 1968)). Subsequent excision would occur by recombination at a site within the viral DNA and a site somewhere on the cellular DNA. A molecule which had lost an SV40 sequence and gained a cellular DNA sequence would result. Presumably, the plaque-purified virus DNA has a low probability of recombining with cell cell DNA (perhaps because genetically homogeneous viral DNA can interact with only a few sites on the cellular genome), but the virus DNA which has acquired host DNA sequences during a previous infection has an enhanced probability of recombining with cellDNA in a subsequent infection. Serial undiluted passage of plaque-purified would thus increase the frequency with which the recombination

event occurs and result in the emergence of progressively larger numbers of viral DNA molecules containing sequences homologous to cell DNA. Viral DNA molecules which have acquired sequences homologous to cell DNA may replicate by complementation with "normal" virus multiplying in the same cell. Hence the increased production of viral DNA containing sequences homologous to cell DNA in cells infected with serially-passaged plaque-purified virus may be due to two separate effects; namely, an increase in the frequency with which the recombination events occur and an increase in the frequency with which replication of the hybrid viral-cell DNA molecules which result from the recombination event occurs.

(2) Viral DNA replication.

The onset of SV40 viral DNA synthesis generally occurs 12-18 hr post-infection, depending on the cell system used. Hirt (1966, 1969) demonstrated that polyoma DNA replicates semi-conservatively and that the replicating DNA molecule forms a structure with two branch points and three branches much like those observed with bacteriophage lambda (Tomizawa & Ogawa, 1968) and ØX174 DNAs (Knippers, Whalley & Sinsheimer, 1969). Replicative intermediates have been detected in both SV40 and polyoma infected cells by labelling appropriate cultures for short periods with ^{3}H-thymidine (Levine, Kyang &

Billheimer, 1970; Bourgaux, Bourgaux-Ramoisoy & Dulbecco, 1969). Levine et al (1970) have shown that replicating SV40 DNA consists of molecules with two branch points , three branches and no visible ends. Two of these branches (replicated branches)were always equal in length and one replicated branch plus the remaining unreplicated portion were equal in length to relaxed circular SV40 DNA (1.66 microns).

Newly-replicated SV40 DNA strands are not covalently linked to the parental DNA strands (Sebring et al, 1971). The absence of a covalent link between parental DNA and newly-replicated DNA excludes the rolling circle model of DNA replication (Gilbert & Dressler, 1968) and indicates instead that replication of the ring proceeds by the mechanism proposed by Cairns (1963, 1966).

The parental strands of most replicating SV40 DNA molecules are covalently linked circles (Sebring et al, 1971; Jaenisch, Mayer & Levine, 1971). This fact presents certain conceptual problems. During replication of a covalently closed duplex, the process of unwinding the two strands would necessarily introduce superhelical turns into the molecule. As replication proceeds, the introduction of these turns would make it progressively more difficult

(and eventually impossible) to unwind the parental strands. To resolve this difficulty, it is necessary to postulate the existence of a swivel in the unreplicated portion of the molecule. Very few replicating SV40 DNA molecules contain such a swivel (Sebring et al, 1971) and this strongly suggests that the swivel is present only intermittently during replication. The simplest possible swivel would be introduced by a single-strand break. The intermittent swivel could then be envisaged as the alternate action of a nicking endonuclease and ligase. Champoux and Dulbecco (1972) have recently demonstrated that nuclei of secondary mouse cells contain an activity capable of untwisting closed circular DNAs with superhelical turns. The enzyme apparently acts by introducing a single-stranded nick into the DNA, forming a DNA-enzyme complex that allows the strands to rotate relative to the helix axis before reversing the action and sealing the break. This enzyme might possibly serve as a swivel during viral DNA replication.

Initiation of replication of the SV40 genome occurs at a specific site (Danna & Nathans, 1972; Thoren et al, 1972) and replication appears to proceed in a bi-directional (Danna & Nathans, 1973; Bourgaux et al, 1971; Fareed et al 1972a) discontinuous (Fareed & Saltzman, 1972) process (Okazaki et al, 1968, 1968a). Concurrent protein synthesis has been shown to be necessary for viral DNA replication (Kit et al, 1969; Levine et al. 1971).

The phenomenon of supercoiling of circular DNA is presumably associated with the ring-closure step. Wang, Baumgarten and Olivera (1967) have suggested three possible causes.

(1) A portion of the molecule is unwound at the time of ring-closure to accomodate the ligase.

(2) The number of base pairs per helical turn is higher in the environment of ring-closure than *in vitro*.

(3) The DNA is associated with a protein core at the time of ring-closure and extraction of the DNA results in the loss of this protein core.

(3) Synthesis of viral RNA.

During productive infection with SV40 there is no reduction in the rate of host cell RNA synthesis and no method has been found to inhibit specifically host RNA metabolism. Therefore, the only way to examine the pattern of viral RNA synthesis in infected cells is by DNA-RNA annealing. Competition hybridisation experiments showed that, during productive infection, certain viral RNA sequences appeared early and were synthesised throughout the virus growth cycle; others were detected only after the onset of viral DNA synthesis (Oda & Dulbecco, 1968; Sauer & Kidwai, 1968; Carp *et al*, 1967; Martin & Axelrod, 1969; Martin, 1970; Tonegawa *et al* 1970; Sauer, 1971).

Westphal (1970) showed that SV40 DNA is an efficient template for RNA synthesis using E. Coli DNA-dependent RNA polymerase. The product (cRNA) is highly asymmetric and can be used to prepare separated strands of the viral DNA. The strand which forms hybrids with cRNA is called E-DNA and the other is called L-DNA.

When the separated strands of SV40 DNA are annealed to RNA extracted from lytically-infected cells at early times after infection, 30% of the sequences of the E-strand enter into hybrid. RNA extracted at late times after infection hybridises to both strands of the DNA. At saturation, 30-35% of the E-strand and about 70% of the L-strand sequences anneal to the RNA (Sambrook, Sharp & Keller, 1972). The early and late subsets of viral RNA are transcribed from different strands of SV40 DNA and possess little or no mutual complementarity.

The factors that bring about the shift in the pattern of viral RNA that occurs as cells pass from the early to the late stages of SV40 infection are unknown. Several explanations are possible.

(1) There may be a change in the physical state of the viral DNA as a consequence of replication, so that a new promoter responsible for the initiation of late RNA synthesis becomes accessible to RNA polymerase.

(2) During the course of infection, there may be a change in the specificity of RNA polymerase which mediates the transition from the early to the late pattern of viral RNA synthesis.

(3) It is known that SV40 DNA becomes integrated into host DNA during lytic infection (Hirai & Defendi, 1972) and it is possible that some classes of RNA are transcribed from the integrated viral RNA sequences under the control of host promoters or terminators.

(4) It may be that primary RNA transcription from SV40 DNA is subsequently modified by post-transcriptional processing events and that changes in such events are responsible for the modulation of viral RNA transcription (Aloni, 1972).

There is evidence that early viral RNA is transcribed from DNA which is integrated with the cell DNA. The size of the heterogeneous nuclear RNA containing SV40 sequences is considerably greater than the segment of the viral genome that is expressed early (Tonegawa et al, 1970; Martin, 1970).

(4) Polypeptide synthesis in SV40 infected cells

SV40 does not repress the synthesis of host cell RNA and proteins and, so far, no inhibitor has been found that

inhibits host cell macromolecular synthesis without simultaneously inhibiting viral replication. It is primarily for this reason that so little is known about the synthesis of SV40 specified polypeptide synthesis, the synthesis of virus induced host proteins or the assembly of virus particles.

Within the first 10 hr of infection, virus-specified RNA and virus - induced T and U antigens can be readily detected (Lewis & Rowe, 1971). Between 12 and 18hr post-infection, viral DNA synthesis begins and by 24 hr, V antigen, associated with assembled capsids, can be detected in the nucleus (Lewis & Rowe, 1971). In addition to the appearance of the virus specific antigens, many enzymes concerned with DNA synthesis increase in activity during the early stage of viral replication (Eckhart, 1968).

During the first 12-20 hr of infection, there is no obvious qualitative change in the species of protein being synthesised compared to the patterns observed in uninfected cells. It would appear that the early SV40 induced proteins are synthesised in relatively small amounts (Anderson & Gesteland, 1972). Shortly after the onset of viral DNA synthesis however, the synthesis of VP1, VP2 and VP3 can be

observed. All three proteins appear at approximately the same time and are synthesised at increasing rates throughout the late portion of the infectious cycle. The synthesis of these three components appears to be contingent upon viral DNA synthesis (Anderson & Gesteland, 1972; Sauer, 1972).

(5) Virus induced synthesis of cellular DNA.

Productive or abortive infection with polyoma or SV40 can stimulate the synthesis of host cell DNA under suitable conditions (Dulbecco et al, 1965; Weil et al, 1965; Winocour et al 1965).

SV40 infection of primary AGMK cells or of CV-1 cells (Kit et al, 1967; Ritzi & Levine,1970) but not of BSC-1 cells (Gershon et al, 1966; Ritzi & Levine, 1970) induces host cell DNA synthesis.

(B) Non-permissive infection

Polyoma induces tumours in newborn hamsters, mice and rats and transforms mouse, hamsters, rat, and bovine cells in culture. SV40 induces tumours in newborn hamsters and transforms hamster, rat, monkey, bovine, porcine and human cells. Tumour cells can be established in culture and cells transformed in vitro produce tumours in appropriate animals.

Transformed cells differ from their normal counterparts in;

(1) altered cell morphology

(2) growth to higher cell densities forming multi-layered colonies

(3) growth in agar

(4) increased growth rate

(5) altered chromosome number or morphology, or both

(6) new antigens

(7) increased trnsplantability in appropriate animals i.e. malignancy

(Black, 1968).

Cells transformed by SV40 or polyoma possess new virus specific molecules including viral gene copies (Sambrook et al, 1968), viral RNA (Westphal & Dulbecco, 1968; Gelb et al, 1971; Ozanne et al, 1973), virus specific antigens (Black et al, 1963).

Since the present work relates only to the process of permissive infection, non-permissive infection will not be further discussed.

Aim of the present work.

The aim of the present work is to determine the form in which the viral DNA exists within the cell and to study the processes which the viral DNA undergoes, especially in relation to the viral assembly process.

MATERIALS AND METHODS

Cell growth and virus infection

BSC-1 cells (Flow Laboratories Ltd., Scotland) were grown in plastic petri dishes (90 mm by 14mm; Nunc Ltd., Denmark) in Eagle's minimal essential medium (Eagle, 1959) containing 10% foetal calf serum (Biocult Ltd., Scotland) in an atmosphere of 5% CO_2 in air. The cells were routinely screened for the presence of mycoplasma by growth on PPLO agar (Biocult Ltd., Scotland; Mycoplasma solid growth medium) in an atmosphere of nitrogen. The cells used in the present study were free from detectable mycoplasma contamination.

The wild-type strain of SV40 virus was plaque-purified and supplied by Dr. J. Williams, Department of Virology, Glasgow University. The SV40 stock used in these experiments was prepared by low multiplicity (0.01 pfu/cell) passage of the virus on confluent BSC-1 cells ; the titre of the stock was 9.6×10^9 pfu/ml.

Confluent monolayer cultures of BSC-1 cells were infected with SV40 virus at a multiplicity of 1 to 100 pfu/cell. After 1 hr of adsorption at 37 C, the cultures were overlaid with 10 ml of Eagle's medium containing 2% foetal calf serum.

Titration of virus

Samples were serially diluted and 0.2 ml samples of each dilution was overlayed on monolayer cultures of BSC-1 cells for 90 min at 37 C. The cells were subsequently overlaid with Eagle's medium containing 2% calf serum in 0.65% noble agar. The cells were fed with Eagle's medium in 0.65% agar on the 9th day and plaques were counted on the 12th day.

Isolation of radioactive SV40 nucleoprotein complex.

Two extraction procedures were employed. Whenever radioactive thymidine was used as the sole isotopic tracer, complexes were extracted from whole infected cells with triton X-100.

To individual dishes of BSC-1 cells infected with SV40, radioactive thymidine was added over the interval 25-50 hr post-infection. After labelling, the cells were washed twice with PBS and SV40 nucleoprotein complexes were extracted by the method of Green et al (1971).

To each dish, 1ml of 0.25% triton X100 (British Drug Houses, Ltd.) in 0.01M tris(hydroxymethyl) aminomethane (tris) -HCl, 0.01M ethylenediaminetetra-acetic acid (EDTA), pH7.9, was added and incubated at 20C for 15 min. NaCl was added to a final concentration of 0.2M. The resulting lysate was carefully scraped into a centrifuge tube and centrifuged at 2,500 rev/min (1,500g) in a MSE Medium centrifuge for 30 min at 4 C. The supernatant material, hereafter referred to as TRITON EXTRACT, was stored at 4 C. Centrifugation at 30,000g did not significantly decrease the yield of acid-precipitable radioactivity in the extract.

When radioactive amino acids were employed, complexes were extracted from isolated nuclei as follows. Monolayer cultures were washed twice with ice-cold phosphate buffered saline (PBS) and the cells were scraped off the dish into

5.0 ml of PBS. The suspension was centrifuged at 1500g for 5 min at 4 C. The cellular pellet was resuspended in 1.0 ml of 0.5% Nonidet NP40 (Shell Chemical Co., U.K.) containing 1mM $MgCl_2$, 0.01M tris-HCl buffer, pH7.9, 0.14M NaCl and incubated for 1 min at 4 C to release nuclei. The mixture was centrifuged at 1500g for 5 min at 4 C and the nuclear pellet was resuspended in 0.5 ml 0.5% NP40 containing 0.01M tris-HCl buffer, pH7.9 and 0.01M EDTA. After 10 min at 4 C, the NaCl concentration was adjusted to 0.2M by the addition of 2.2M NaCl. After 5 min at 4 C, the sample was centrifuged at 2000g for 10 min at 4 C and the supernatant material, which contained the complex, was removed and stored at 4 C.

Preparation of labelled SV40 virus

Two monolayer cultures of BSC-1 cells were infected with 0.01 pfu/cell of SV40 virus and after 80 hr, ^{3}H-leucine or ^{3}H-lysine or ^{14}C-protein-hydrolysate (200 µCi/dish, 200 µCi/dish, and 25 µCi/dish respectively) was added. At 160 hr post-infection, the cells were suspended in the growth medium by scraping and stored at 4 C for 1 hr. The mixture was centrifuged at 15000g for 30 min then the cells were suspended in 2.0ml PBS and made 1% with respect to sodium deoxycholate. Deoxyribonuclease and ribonuclease A (Sigma Chemical Corp.) were added to final concentrations of 40 µg/ml and 12 µg/ml respectively.

The mixture was then dispersed by sonic vibration then incubated at 37 C for 30 min. Cellular debris was removed by centrifugation at 15,000g for 30 min at 4 C. The virus-containing supernatant was underlayed with 2 ml of saturated KBr solution and centrifuged for 3 hr at 40,000 rev/min in the Spinco SW50.1 rotor at 20 C. The fractions corresponding to the lower visible band (SV40 virus) or to the upper visible band ('empty' shells) were separately pooled and layered on to 3.5 ml CsCl (density 1.36 g/ml) and centrifuged for 18 hr in the Spinco SW56Ti rotor at 40,000 rev/min at 20 C. The visible bands corresponding to SV40 virus (density 1.34 g/ml) or empty shells (density 1.30 g/ml) were collected and dialysed for 2 hr against PBS at 4 C.

Typical profiles obtained for ^{3}H -thymidine labelled virus (A), for ^{3}H-leucine labelled virus (B) and for ^{3}H-leucine labelled 'empty' shells are shown in Fig (i). Profiles obtained on analysis of similarly labelled mock-infected cells are shown in each case (o ---o).

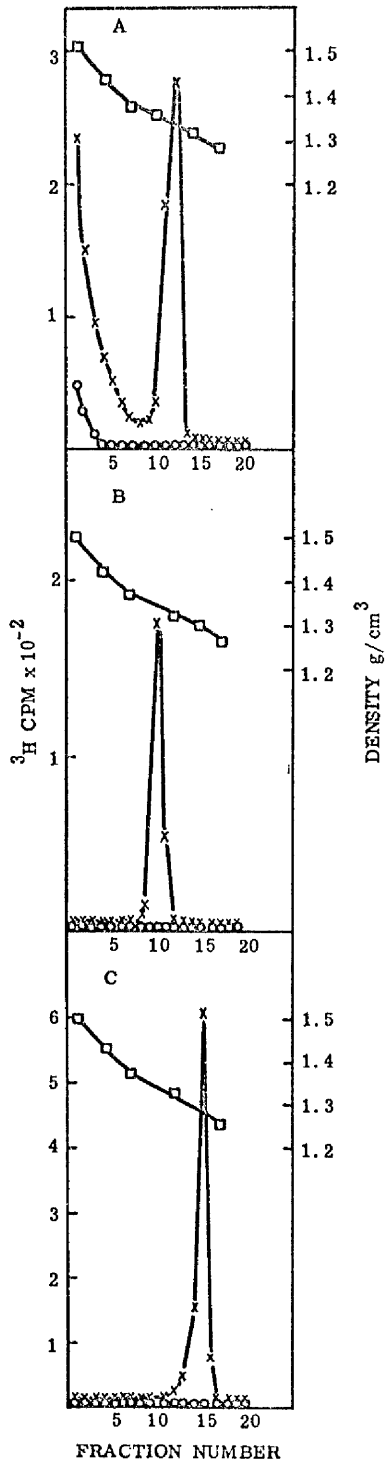


Fig. 1.

Preparation of labelled SV40 DNA

Using ^{32}P-phosphate

One hundred μCi of ^{32}P-inorganic phosphate were added to single petri dishes of monolayer BSC-1 cells infected with SV40 at 20 hr after infection , and thereafter at 12 hr intervals. Three days after infection, SV40 DNA was extracted with sodium dodecyl sulphate (SDS) by the method of Hirt (1967). The SV40 DNA extract was made 1.0M in CsCl and stored at 0 C for 30 min. Caesium dodecyl sulphate was removed by centrifugation at 14,000g for 10 min at 2 C. The DNA was centrifuged in 5.0 ml CsCl (density 1.52 g/ml) - propidium di-iodide (PDI; 500 μg/ml; Calbiochem, Los Angeles, California) and centrifuged for 40 hr in a SW50L rotor at 40,000 rev/min at 20 C (Hudson et al, 1969). The tubes were then punctured and 6 drop fractions were collected. Five μl aliquots were applied to 2.5 cm Whatman 3MM filter discs and assayed for radioactivity as described below. The fractions which contained supercoiled SV40 DNA were pooled and dialysed twice against PBS for 1 hr. PDI was removed by repeated extraction with isoamyl alcohol. Purified DNA was stored at -20 C.

Using ^{14}C-thymidine

BSC-1 cells were infected with SV40 virus (100 pfu/cell) and labelled 26-44 hr post-infection with ^{14}C-thymidine (1.25 μCi/ml) prior to extractionof DNA by the method of Hirt (1967). The Hirt supernatant was adjusted to 1.0M in CsCl, stored at 4 C for 30 min and centrifuged at 14,000g for 10 min at 4 C,. The resulting supernatant material was dialysed for 2 hr against 0.01M tris buffer, pH7.9, 0.01M EDTA, 0.2M NaCl at 4 C then stored at -20 C.

Centrifugation techniques and isotope measurements

Linear sucrose gradients (5-20%) were prepared containing 0.01M tris buffer, pH7.9, 0.01M EDTA, 0.2M NaCl. The volume of the gradient was 4.0 ml when the Spinco SW56Ti rotor was used, 13.0 ml when the Spinco SW40Ti rotor was used, and 5.0 ml when either the SW50L, SW50.1 or SW65L rotor was used. Individual conditions of centrifugation are detailed in the figure legends.

Velocity sedimentation analysis on CsCl was performed by layering 0.2 ml samples on 3.0 ml CsCl (density 1.50 g/ml) in 0.01M tris-HCl at pH7.9. Samples were centrifuged in a SW50L rotor for 3 hr at 40,000 rev/min at 20 C and harvested as described below.

Equilibrium dye-buoyant centrifugation was performed by adding 0.2 ml samples to make a final volume of 3.2 ml CsCl

(density 1.52 g/ml)-PDI (500 µg/ml). Centrifugation was carried out in a SW50L or SW50.1 rotor at 40,000 rev/min at 20 C.

Gradients were harvested by direct tube puncture, and 2 drop fractions were collected on 2.5 cm Whatman 3MM filter discs. The discs were washed three times in ice-cold 10% trichloroacetic acid and dried in ethanol-ether, after which they were counted in either a Phillips liquid scintillation analyser or a Nuclear Chicago Isocap 300 liquid scintillation system. The scintillation fluid consisted of 0.5% 2,5-diphenyl-oxazole in toluene.

Electrophoresis in SDS-polyacrylamide gels.

0.1-0.2 ml samples were adjusted to 1.0% SDS, 5.0% 2-mercaptoethanol (ME) and 8M urea then incubated at 70 C for 1 hr.

Acrylamide gels (10%; 7mm x 70mm) had an acrylamide: bis-acrylamide ratio of 50: 1 and contained 0.07% tetramethylethylenediamine (Koch), 0,1M phosphate buffer, pH7.2, 0.1% SDS. Electrophoresis was carried out for 3.5 hr at 16 mA/gel. The gels were sliced laterally and prepared for scintillation counting as described below.

Gel slices were digested by incubation overnight at 60 C with 0.3 ml of hydrogen peroxide. Scintillation fluid (10 ml), composed of 3 parts by volume of toluene containing 0.5% 2,5-diphenyloxazole and 2 parts 2-methoxyethanol, was added

and samples were counted in a Phillips liquid scintillation analyser equipped with computerised quench correction.

The molecular weights of proteins were determined by the method of Shapiro et al,(1967) by comparing their electrophoretic mobilities with the migration of a series of proteins of known molecular weights. Bovine serum albumin (mol wt. 66,500), carboxypeptidase (mol wt. 34,000), chymotrypsin (mol wt. 24,500) and cytochrome C (mol wt. 12400) were used as standards.

Buoyant density of SV40 DNA-protein complex

Triton extract (0.15 ml), prepared from SV40 virus infected cells labelled with ^{3}H-thymidine, was mixed with 0.05 ml of ^{14}C-SV40 DNA and made 1% with respect to glutaraldehyde (Koch) in 0.01M EDTA, 0.2M NaCl, 0.01M tris-HCl buffer, pH7.9, and incubated for 15 min at 20 C. 0.1 ml of the mixture was added to 3.0 ml of CsCl (density 1.50 g/ml) and centrifuged for 40 hr at 40,000 rev/min in the Spinco SW50.1 rotor. Fractions, collected by tube puncture, were assayed for radioactivity and aliquots used for density determination.

Radiochemicals

All radiochemicals were obtained from the Radiochemical Centre, Amersham, England at the following specific activities; thymidine -2-C^{14}, 62 mCi/mmole; uridine -6-H^{3}, 5 Ci/mmole;

thymidine-6-H^3, 26Ci/mmole; L-leucine-4,5-H^3, 52Ci/mmole; protein hydrolysate-C^{14}, 54mCi/matom carbon; L-lysine-4,5-H^3, 5.5 Ci/mmole; ^{32}P-inorganic phosphate, 50Ci/mg.

Drugs

Puromycin was obtained from Serva, Heidelberg. Actinomycin D and arabinofuranosylcytosine (ara-C) were purchased from Sigma Chemical Co., London.

Enzymes

Ribonuclease (RNase; Sigma Chemical Co., Ltd, London), 200 µg/ml in phosphate buffered saline (PBS) was heated to 85 C for 20 min to inactivate latent deoxyribonuclease activity. Pronase (Calbiochem, Los Angeles, California), 200 µg/ml in PBS, was autodigested for 2 hr at 37 C to destroy latent DNase activity. Deoxyribonuclease was obtained from Sigma Chemical Co., Ltd., London.

RESULTS

Isolation of SV40 Nucleoprotein Complex

Twenty-five hr. after infection of BSC-1 cells with SV40 virus, ^{3}H-thymidine was added for 5 hr. The cells were then treated with 0.25% triton and a soluble supernatant extract was prepared as described in the preceding section. A 0.2 ml sample of the extract was mixed with ^{32}P-labelled 21S (supercoiled) SV40 DNA and centrifuged through a linear 5-20% sucrose gradient. The results of this experiment are presented in Fig.1.

The triton extract contained a component which sedimented more rapidly than purified ^{32}P-labelled 21S SV40 DNA and which accounted for all the radioactivity present in the sample. The rapidly sedimenting material had a sedimentation coefficient at the peak maximum of 44S, compared with the value of 21S for the SV40 DNA marker. No ^{3}H-labelled material was detected in the region of 21S SV40 DNA. In mock-infected BSC-1 cells labelled in a similar manner, negligible amounts of acid-precipitable radioactivity were present in triton extracts.

When a sample of triton extract, mixed with ^{32}P-labelled 21S SV40 DNA, was analysed by velocity centrifugation on CsCl (Fig.1B), the ^{3}H-radioactivity was found to co-sediment with the ^{32}P-labelled marker SV40 DNA. Furthermore, on analysis by CsCl propidium di-iodide buoyant centrifugation, the ^{3}H-radioactivity was observed to have a buoyant density corresponding to the closed circular form of SV40 DNA (Fig.2).

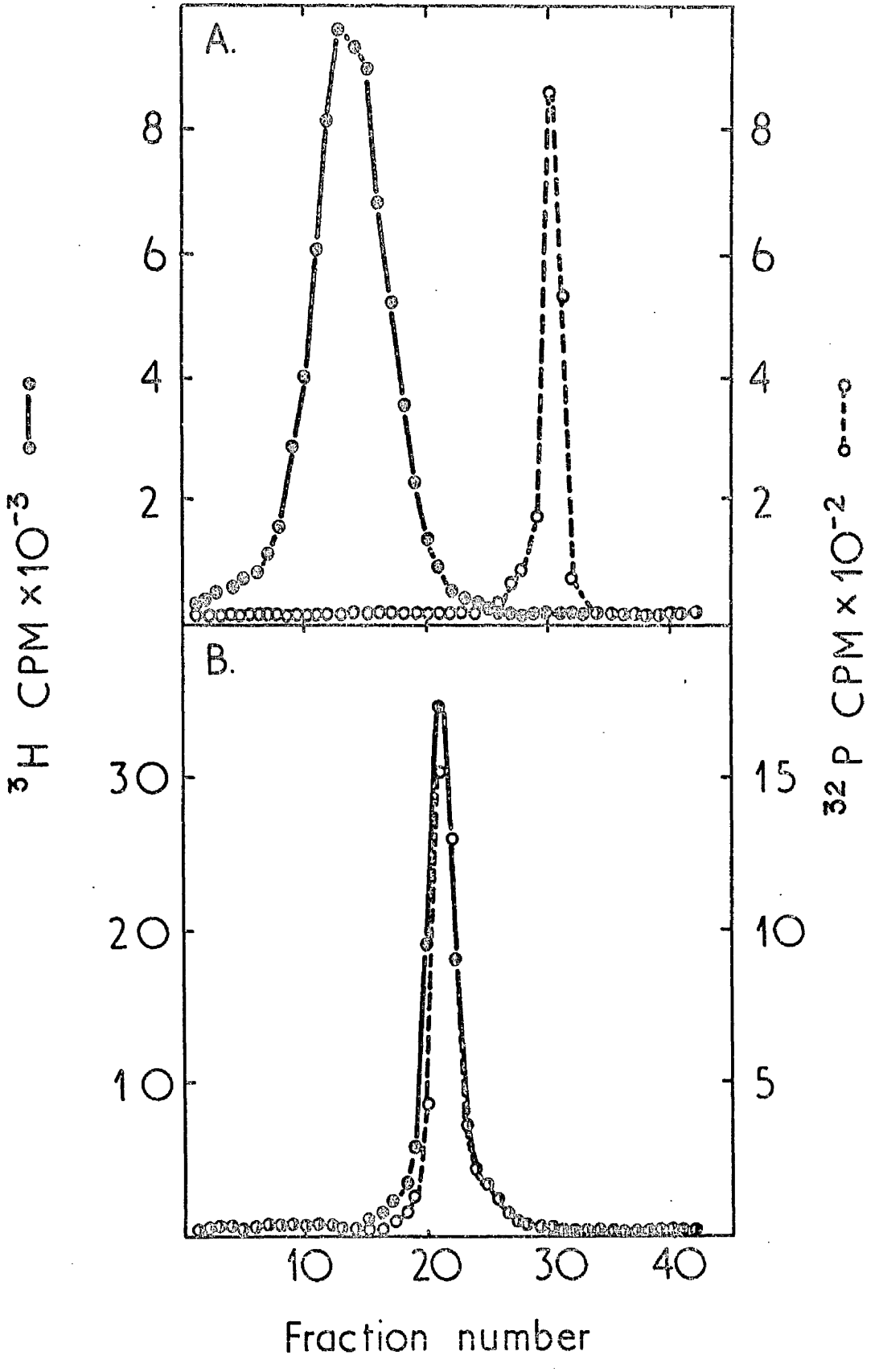
These results demonstrate that the 44S material, observed in 5 hr labelled triton extracts by sedimentation analysis on sucrose gradients, contains 21S SV40 DNA, which is associated with other macromolecular components.

In an attempt to test for non-specific association of SV40 DNA with components in the triton extract, ^{32}P-labelled 21S SV40 DNA was incubated with a sample of triton extract for 1 hr at 20 C. The sedimentation properties of the 21S SV40 DNA in sucrose gradients were unaltered after such treatment, and no complexes were present. Since this experiment cannot duplicate precisely the conditions in the cell at the time of extracting, some form of non-specific interaction cannot be excluded at this point.

Evidence that Protein is Present in the Complex.

In order to determine the nature of the material complexed to SV40 DNA in the 44S component, the following experiments were performed. A 0.2ml sample of the triton extract, obtained

Fig.1. Sedimentation analysis of triton extract from SV40 infected cells. The cells were labelled 25 hr. post-infection with ^{3}H-thymidine for 5 hr. before harvesting. ^{32}P-labelled 21S SV40 DNA was added as a marker. (A) Centrifugation on a 5-20% sucrose gradient was for 2 hr. in a SW50L rotor at 36,000 rev/min at 4 C. (B) The sample was layered on 3 ml CsCl (density 1.50 $g.ml^{-1}$) and centrifuged for 3 hr. in a SW50L rotor at 40,000 rev/min at 20 C.
The multiplicity of viral input was 10 pfu/cell and the ^{3}H-thymidine concentration was 5 µCi/ml.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Fraction number
^{3}H CPM $\times 10^{-3}$
^{32}P CPM $\times 10^{-2}$

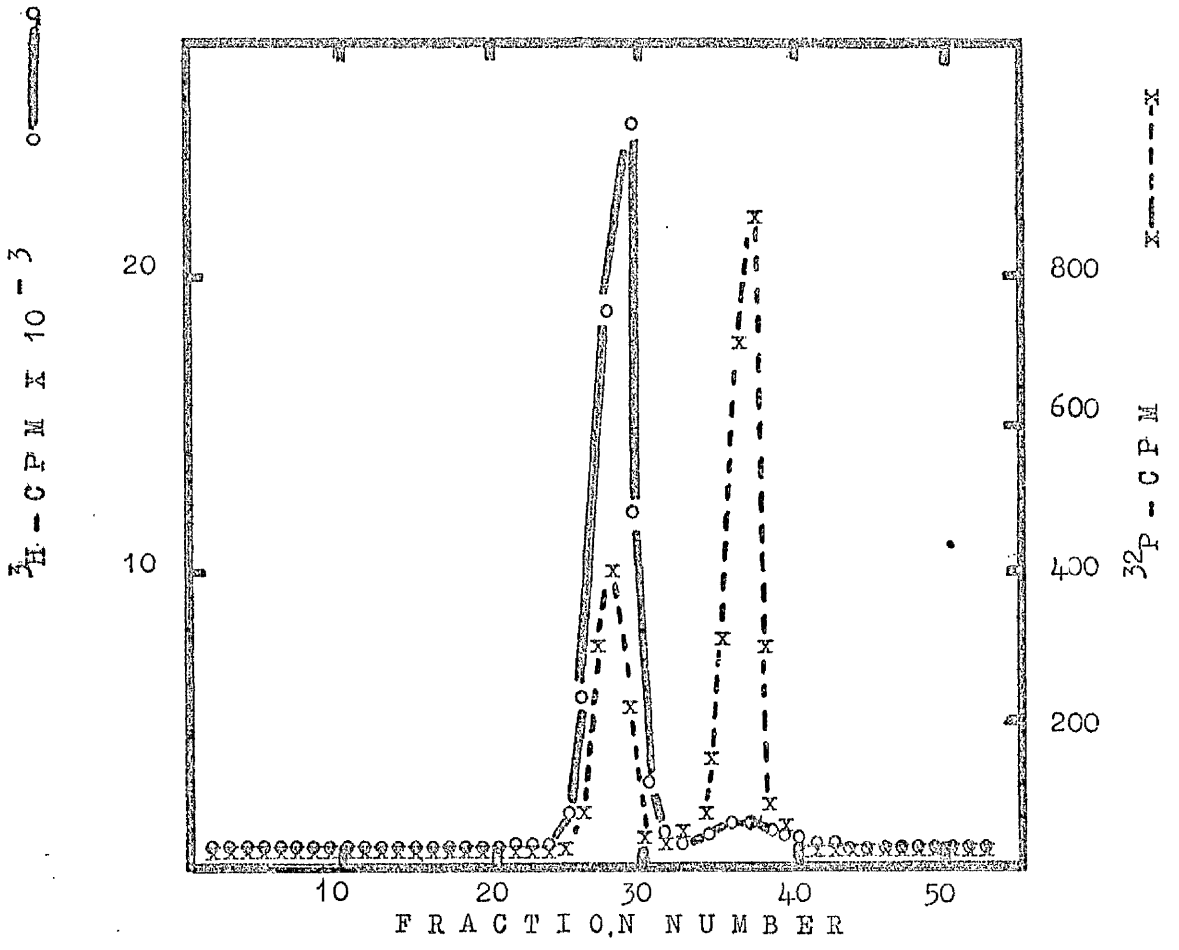


Fig.2. Dye-buoyant analysis of triton extract from SV40 infected cells. Triton extract was prepared from SV40-infected BSC-1 cells as described in Fig.1. A 0.2 ml sample of extract was mixed with ^{32}P-labelled SV40 DNA (form 1 and form 2) and centrifuged in CsCl (density 1.52 g.ml^{-1}) in PDI (500 ug/ml) for 40 hr. in a Spinco SW50L rotor at 40,000 rev/min at 20 C

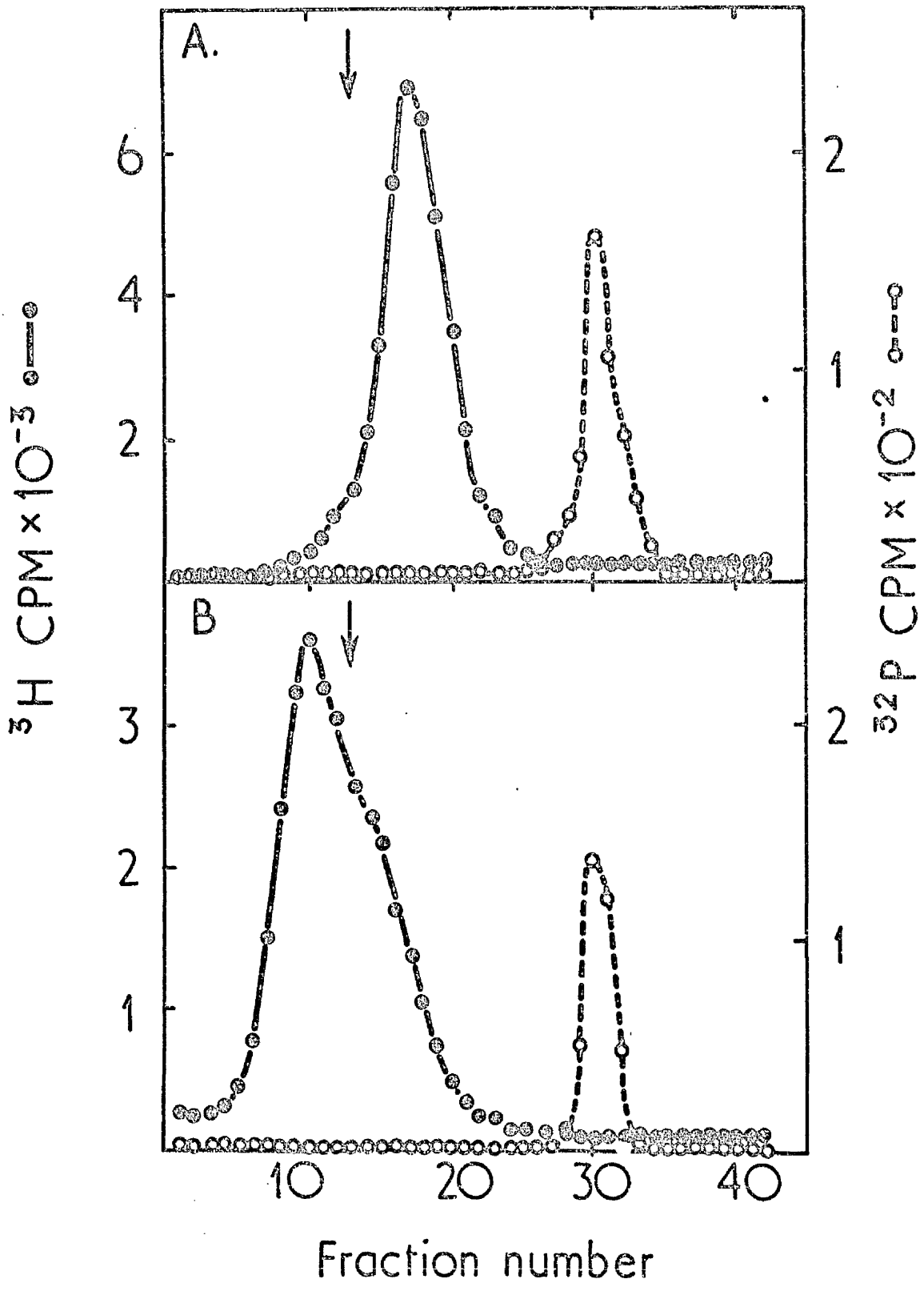
in the previous experiment, was incubated with heat-treated RNase (10 µg/ml) for 15 min at 20 C. A further sample of the triton extract was incubated with pronase (10 µg/ml) under the same conditions. These two samples were separately mixed with ^{32}P-labelled 21S SV40 DNA and centrifuged through 5-20% sucrose gradients as previously described. The results of this experiment are shown in Fig. 3.

Digestion of the complex with pronase under the conditions described gave rise to a component (Fig. 3A) with a sedimentation coefficient of 39S and more prolonged incubation led to a further reduction in sedimentation coefficient (Fig.4).

Incubation of the complex with RNase (Fig.3B) did not decrease the rate of sedimentation of the complex and it is evident therefore that the complex did not contain significant amounts of RNA. A slight increase in the average sedimentation coefficient (48S) was observed and is attributed to association of the complex with RNase. A similar effect has been reported by Green et al (1971).

Double label experiments were performed using ^{3}H-thymidine and ^{14}C-protein hydrolysate to provide additional evidence that the material associated with the viral DNA was protein (Fig.5). BSC-1 cells infected with SV40 virus were labelled 27 hr post-infection with ^{3}H-thymidine and ^{14}C-protein

Fig.3. (A) Sedimentation of triton extract after incubation with pronase, which was added to a final concentration of 10 µg/ml and the mixture incubated for 15 min at 20 C. (B) Sedimentation of triton extract after treatment with ribonuclease, which was added to a final concentration of 10 µg/ml and the mixture was incubated for 15 min at 20 C. The triton extract was prepared from infected BSC-1 cells as described in Fig.1 and the procedures for sucrose gradient analysis are presented in the legend for Fig.1(A). The arrow indicates the position of the complex in untreated triton extract.


A.
B
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1
2
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20
30
40
Fraction number
^{3}H CPM x 10^{-3}
^{32}P CPM x 10^{-2}

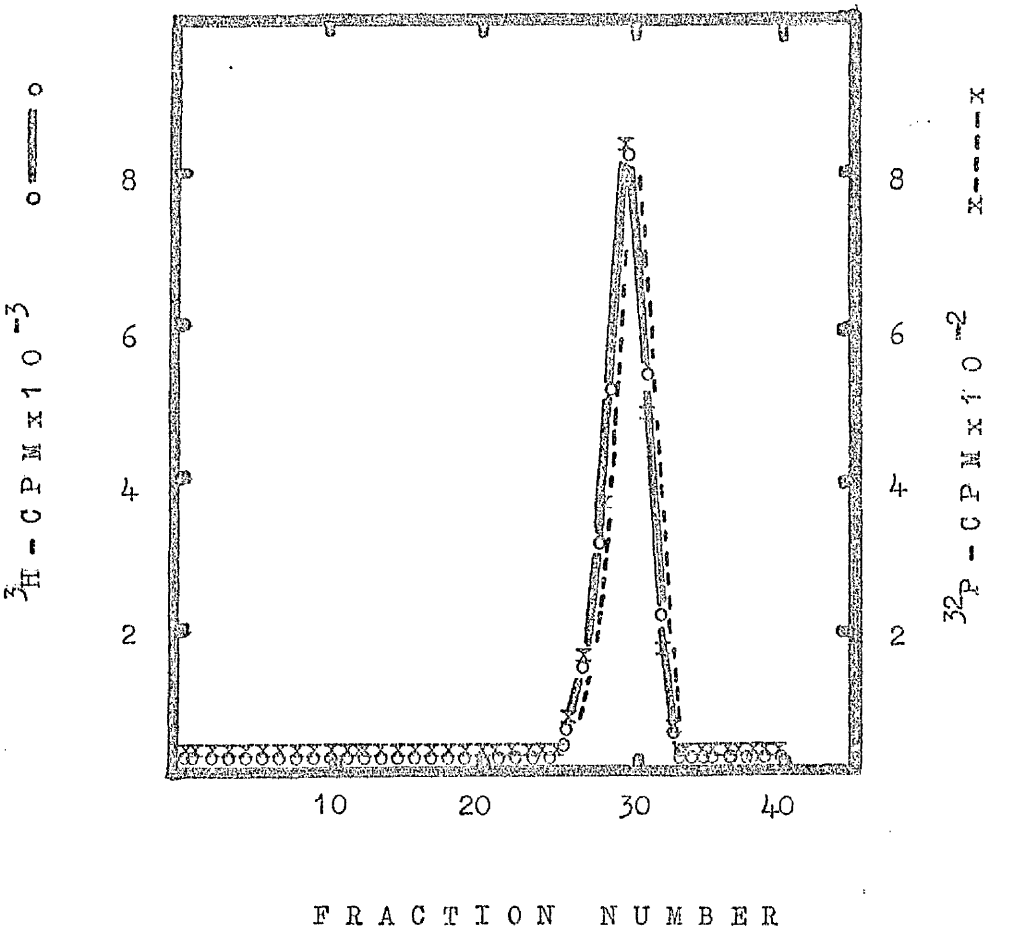
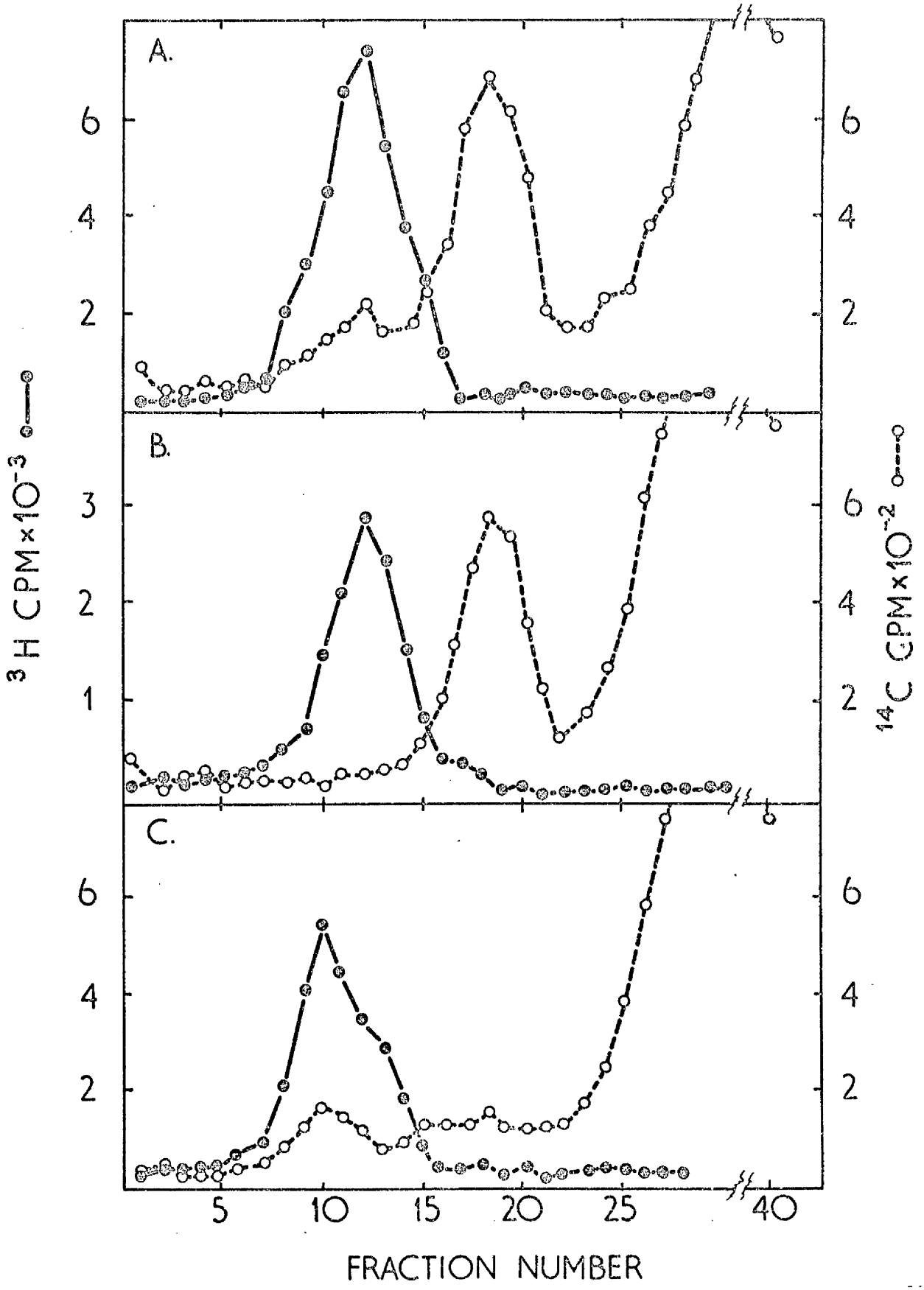


Fig.4. Sedimentation of triton extract after prolonged exposure to pronase. Triton extract was prepared as described in Fig.1 and pronase was added to a final concentration of 10 µg/ml. The mixture was incubated for 2 hr. at 37 C. A 0.2 ml aliquot was subsequently mixed with ^{32}P-labelled 21S SV40 DNA and analysed by sucrose gradient centrifugation as described in Fig.1.

hydrolysate for 3 hr and triton extracts were prepared. Aliquots (0.5 ml) were centrifuged through a linear 5-20% sucrose gradient and the results are shown in Fig. 5A. A small amount of ^{14}C-labelled material was observed to cosediment with ^{3}H-labelled complex. In addition, a ^{14}C-labelled component was observed with a sedimentation coefficient of 40S. A large amount of ^{14}C-labelled material, probably free protein, remained at the top of the gradient. Triton extracts of mock-infected BSC-1 cells similarly labelled with ^{14}C-protein hydrolysate were prepared and a 0.5 ml aliquot was mixed with 0.1 ml of triton extract from infected cells labelled with ^{3}H-thymidine as described in Fig. 1. The mixture was centrifuged on a linear 5-20% sucrose gradient and the results are shown in Fig. 5B. A ^{14}C-labelled 40S component was observed along with a large amount of ^{14}C-labelled material at the top of the gradient. It was concluded that the 40S component observed in Fig. 5A is probably of cellular origin and does not arise as a result of virus infection. Since it was probable that the 40S component contained RNA, 0.5 ml aliquots of the triton extract analysed in Fig. 3A were incubated with 20 µg/ml RNase for 1 hr at 37 C, then analysed on linear 5-20% sucrose gradients (Fig. 5C). The 40S component observed in Fig. 5A was no longer present but the ^{14}C-labelled material associated with the ^{3}H-labelled 44S complex was unaffected by

Fig.5. Sedimentation of triton extracts from SV40 - infected BSC-1 cells and mock - infected BSC-1 cells after labelling with ^{14}C- protein hydrolysate. (A) Infected cells 27 hr. post-infection were labelled for 3 hr. with ^{14}C-protein hydrolysate and ^{3}H-thymidine and triton extracts were prepared. (B) Mock-infected BSC-1 cells were labelled in parallel with ^{14}C-protein hydrolysate and a triton extract was prepared. (C) The double-labelled extract from infected cells described in part (A) above was incubated with 20 µg/ml ribonuclease for 1 hr. at 37 C. All samples were centrifuged in 5-20% linear sucrose gradients for 3.25 hr. in a Spinco SW40Ti rotor at 40,000 rev/min in a Beckman L2-65B ultracentrifuge at 4 C. The first 30 fractions only out of a total of 40 collected are shown due to the large amount of ^{14}C-labelled material (10,000 cpm) at the top of the gradient.
The multiplicity of viral input was 10 pfu/cell. The ^{3}H-thymidine and ^{14}C-protein hydrolysate concentrations employed were 5 µCi/ml and 7.5 µCi/ml respectively.


A.
B.
C.
^{3}H CPM×10^{-3}
^{14}C CPM×10^{-2}
FRACTION NUMBER
5
10
15
20
25
40

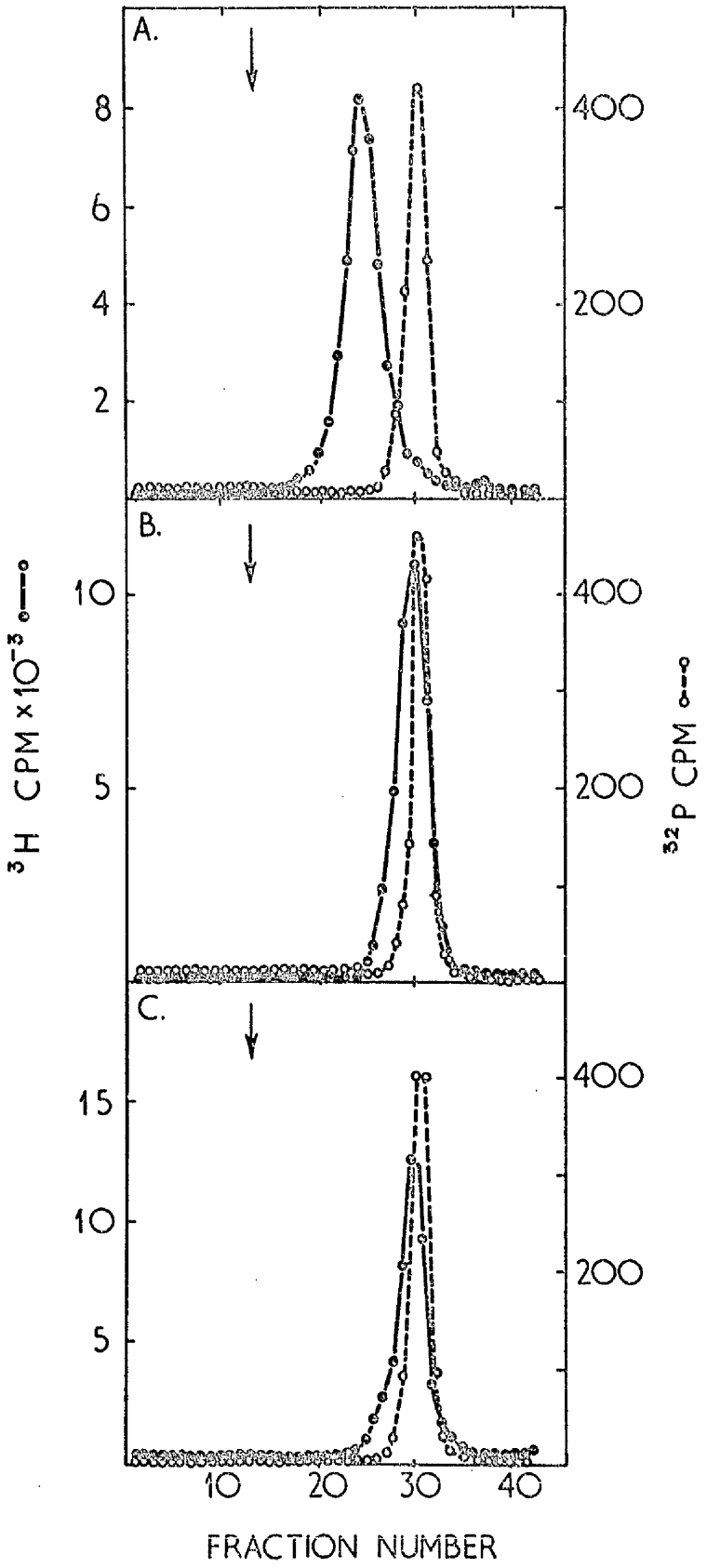
this treatment. The 40S component is probably a ribonucleoprotein of cellular origin whose presence in triton extracts is independent of SV40 infection. The 44S ^{14}C-labelled material occurs only in SV40-infected cells and is probably due to protein associated with the viral DNA. These results indicate that in the complex extracted with triton, 21S SV40 DNA is probably associated with protein.

The effect of salt concentration and detergent treatment on the sedimentation properties of the nucleoprotein complex is shown in Fig. 6. When the triton extract was treated with 0.5 M NaCl, a component was observed on sucrose gradients with a sedimentation coefficient of 29S (Fig. 6A). Treatment with either 1.0 M NaCl (Fig. 6B) or with 0.5% SDS (Fig. 6C) yielded components which sedimented only slightly in advance of the 21S SV40 DNA marker, indicating effectively complete dissociation of the complex. Binding of protein to DNA in the complex is sensitive to variation in salt concentration and this behaviour is similar to that of the polyoma DNA-protein complex in infected ME cells (Green et al, 1971).

Treatment of the complex with 0.25% deoxycholate for 1 hour caused partial dissociation of the components of the complex (Fig. 7A).

After such treatment, the complex was observed to sediment heterogeneously between 35S and 21S. However more prolonged

Fig.6. Sedimentation of triton extracts after treatment in dissociating conditions. (A) The triton extract was adjusted to 0.5 M NaCl and incubated for 15 min at 20 C. The sample was centrifuged in a 5-20% sucrose gradient adjusted to 0.5 M in NaCl. (B) The triton extract was adjusted to 1.0 M in NaCl and incubated for 15 min at 20 C. The sample was centrifuged in a 5-20% sucrose gradient adjusted to 1.0 M in NaCl. (C) The triton extract was made 0.5% with respect to sodium dodecyl sulphate and incubated for 1 hr. at 37 C. The sample was centrifuged in a 5-20% sucrose gradient. The triton extract was prepared from SV40-infected BSC-1 cells as described in Fig.1. ^{32}P-labelled 21S SV40 DNA was added as marker and centrifugation was for 2 hr. in a Spinco SW50L rotor at 36,000 rev/min at 4 C. The arrow indicates the position of the complex in untreated triton extracts.


A.
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400
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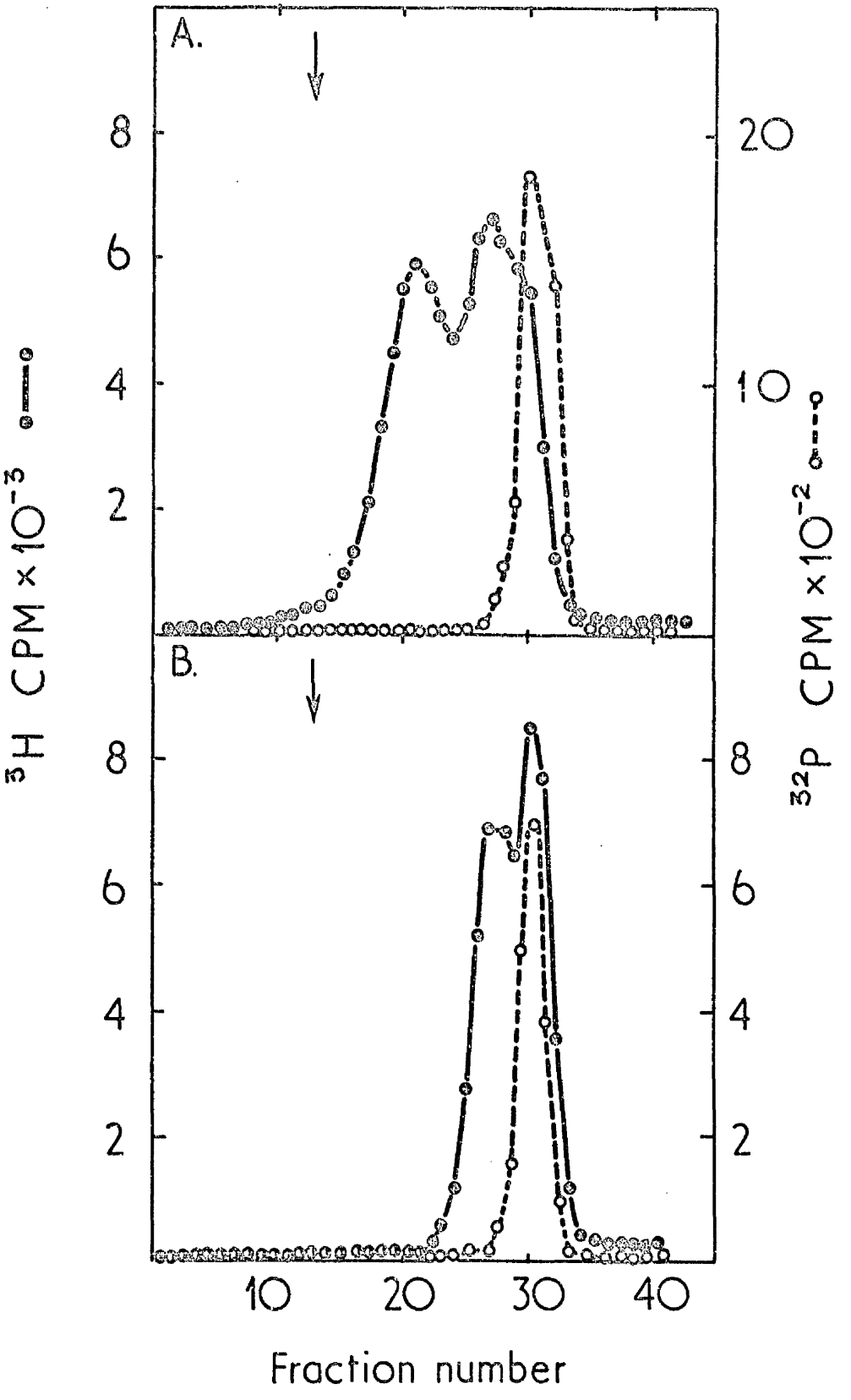
30
40
FRACTION NUMBER
^{3}H CPM x 10^{-3}
^{32}P CPM

Fig.7. Sedimentation analysis of tritom extract after treatment with deoxycholate. Triton extract, prepared as described in Fig.1 was made 0.25% with respect to sodium deoxycholate and incubated at 20 C for either 1 hr. (A) or 18 hr (B). Thereafter, the complex was mixed with ^{32}P-labelled 21S SV40 DNA and centrifuged through a 5-20% sucrose gradient as described in Fig.1.


A.
B.
8
6
4
2
20
10
8
6
4
2
^{3}H CPM $\times 10^{-3}$
^{32}P CPM $\times 10^{-2}$
10
20
30
40
Fraction number

treatment dissociated the complex still further to a mixture of 25S complex and 21S DNA (Fig. 7B).

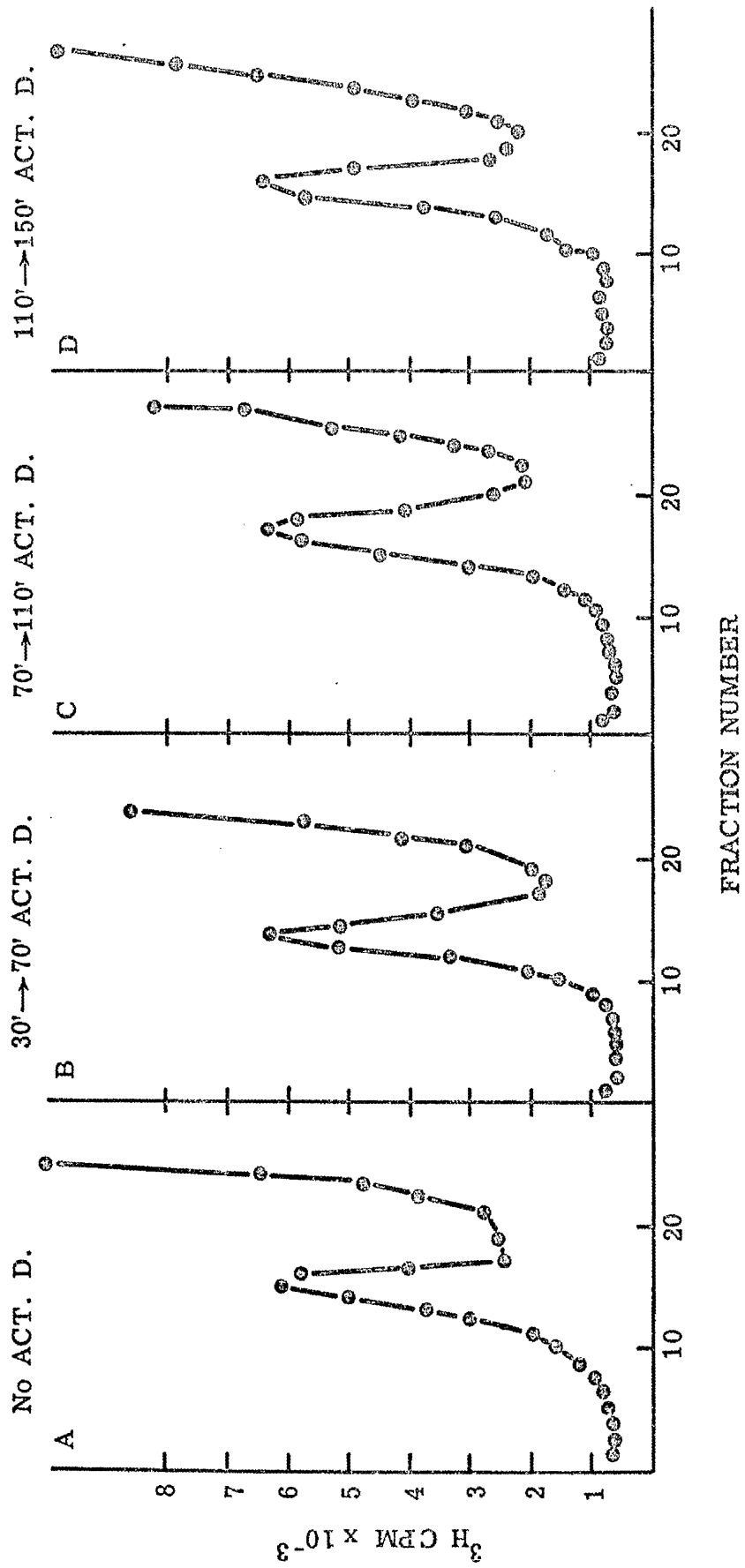
Analysis of the polypeptide constituents of the complex.

Analysis of the polypeptide constituents of SV40 DNA-protein complexes extractable with Triton X-100 is complicated by the presence in the extracts of a protein-containing component with a sedimentation coefficient (40S) close to that of the complex. This component was found to be of cellular origin and did not arise as a result of virus infection. It was sensitive to ribonuclease treatment, but the enzyme bound to the SV40 DNA-protein complex and prolonged incubation resulted in extensive losses due to nuclease activity in the Triton extracts.

In order to determine the sensitivity of the 40S component to actinomycin D, 3 monolayer cultures of BSC-1 cells were treated with 0.4 μg/ml of actinomycin D and at varying times thereafter were pulsed with ^{3}H-leucine for 40 min periods. At the end of the pulse period, each culture was extracted with triton X-100 and the extracts thus obtained were analysed by sucrose gradient centrifugation. In eachcase, the amount of ^{3}H-radioactivity incorporated into the 40S component was approximately equivalent to that observed on analysis of an extract obtained from a culture labelled with ^{3}H-leucine in the absence of actinomycin D (Fig. 8.). Incorporation of radioactive leucine was therefore insensitive to

Fig.8. Effect of actinomycin D on incorporation of ^{3}H-leucine into the 40S ribonucleoprotein complex of triton extracts. The medium was removed from 4 resting monolayer cultures of BSC-1 cells and replaced with warm Eagle's medium containing 0.4 µg/ml of actinomycin D, or in the case of one of the cultures, with Eagle's medium containing no actinomycin D (A). 30 min later, the medium was removed from both a drug-treated culture (B) and the single non drug-treated culture (A) and replaced with warm leucine-free Eagle's medium containing ^{3}H-leucine (20 µCi/ml) and either actinomycin D or no actinomycin D. After a 40 min exposure to isotope, the cultures were extracted with triton X-100. The 2 remaining drug-treated cultures were similarly labelled with ^{3}H-leucine in the presence of actinomycin D for 40 min periods at 70min (C) and 110 min (D) after the addition of drug and triton extracts were prepared from each culture.

0.2 ml samples of each extract were analysed by velocity centrifugation through linear 5-20% sucrose gradients at 55,000 rev/min for 50 min in the Spinco SW56Ti rotor at 4 C.


No ACT. D.
30'→70' ACT. D.
70'→110' ACT. D.
110'→150' ACT. D.
A
B
C
D
^{3}H CPM x 10^{-3}
FRACTION NUMBER

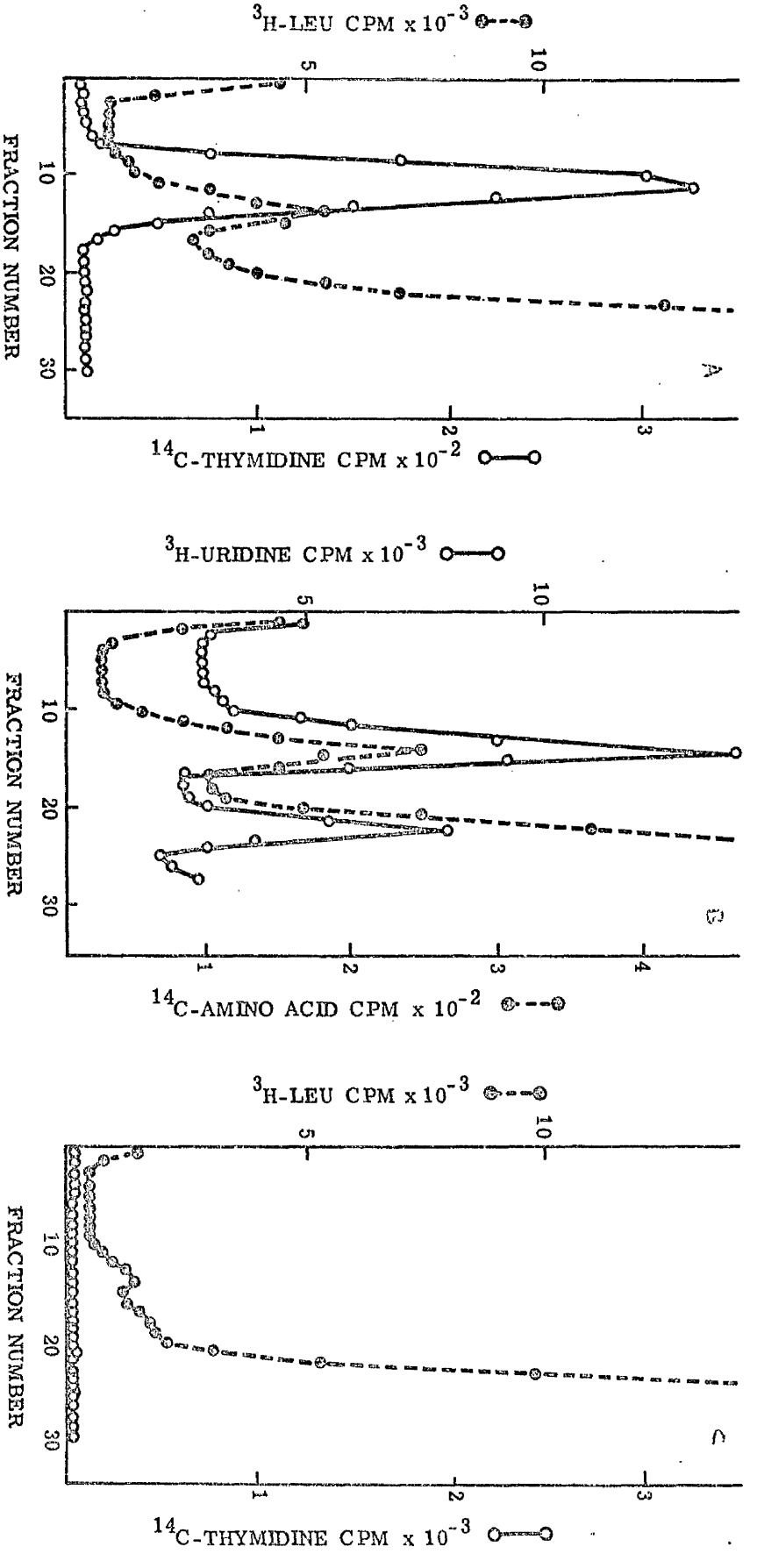
actinomycin D under the conditions employed.

The first step in overcoming the problems associated with the presence of the 40S component has been to develop a nuclear extraction procedure which reduced the level of 40S component present, compared with the Triton extracts. Uninfected resting cultures of BSC-1 cells were incubated at 37°C in leucine-free Eagle's medium to which ^{3}H-leucine was added. After 3 hr, a nuclear extract was prepared and a sample was mixed with an aliquot of Triton extract from SV40 virus-infected BSC-1 cells, labelled 28-44 hr post-infection with ^{14}C-thymidine. The mixture was centrifuged through a linear 5-20% sucrose gradient (Fig.9A). A component with sedimentation coefficient 40S was observed compared to the 44S marker. Labelling with ^{3}H-uridine and ^{14}C-protein hydrolysate demonstrated the presence of RNA in the 40S material (Fig. 9B). The amount of 40S component obtained by the nuclear extraction procedure was less than 10% of that obtained by the Triton X-100 procedure.

To determine the sensitivity of the nuclear 40S component to actinomycin D, a monolayer culture of BSC-1 cells was treated with 0.1 µg/ml actinomycin D for 1 hr. The medium was replaced with leucine-free Eagle's medium containing ^{3}H-leucine plus 0.1 µg/ml actinomycin D and the cells were incubated for 3 hr. A nuclear extract was prepared and sucrose gradient analysis (Fig. 9C) revealed

Fig.9. Sedimentation analysis of nuclear extracts from uninfected BSC-1 cells. The medium was removed from 3 resting monolayer cultures of BSC-1 cells and replaced with warm Eagle's medium, or in the case of one of the cultures, with Eagle's medium containing actinomycin D (0.1 µg/ml). After an incubation period of 1 hr, the medium was removed and replaced with either warm leucine-free Eagle's medium containing ^{3}H-leucine (50 µCi/ml) (A) or with leucine-free Eagle's medium containing both ^{3}H-leucine (50 µCi/ml) and actinomycin D (0.1 µg/ml) (C), or with Eagle's medium containing ^{3}H-uridine (25 µCi/ml) and ^{14}C-protein hydrolysate (10 µCi/ml) (B). After a 3 hr exposure to isotope, nuclear extracts were prepared in each case.

In the case of extract (A), 0.1 ml of a triton extract prepared from SV40 virus-infected BSC-1 cells labelled with ^{14}C-thymidine was added as a marker for the SV40 nucleoprotein complex. The extracts were thereafter centrifuged in 5-20% linear sucrose gradients for 3.5 hr in the SW40Ti rotor of a Beckman L2-65B ultracentrifuge at 40,000 rev/min at 4C. Fractions were collected and 50 µl aliquots of each were assayed for radioactivity.


^{3}H-LEU CPM x 10^{-3}
FRACTION NUMBER
^{14}C-THYMIDINE CPM x 10^{-2}
A
^{3}H-URIDINE CPM x 10^{-3}
FRACTION NUMBER
^{14}C-AMINO ACID CPM x 10^{-2}
B
^{3}H-LEU CPM x 10^{-3}
FRACTION NUMBER
^{14}C-THYMIDINE CPM x 10^{-3}
C

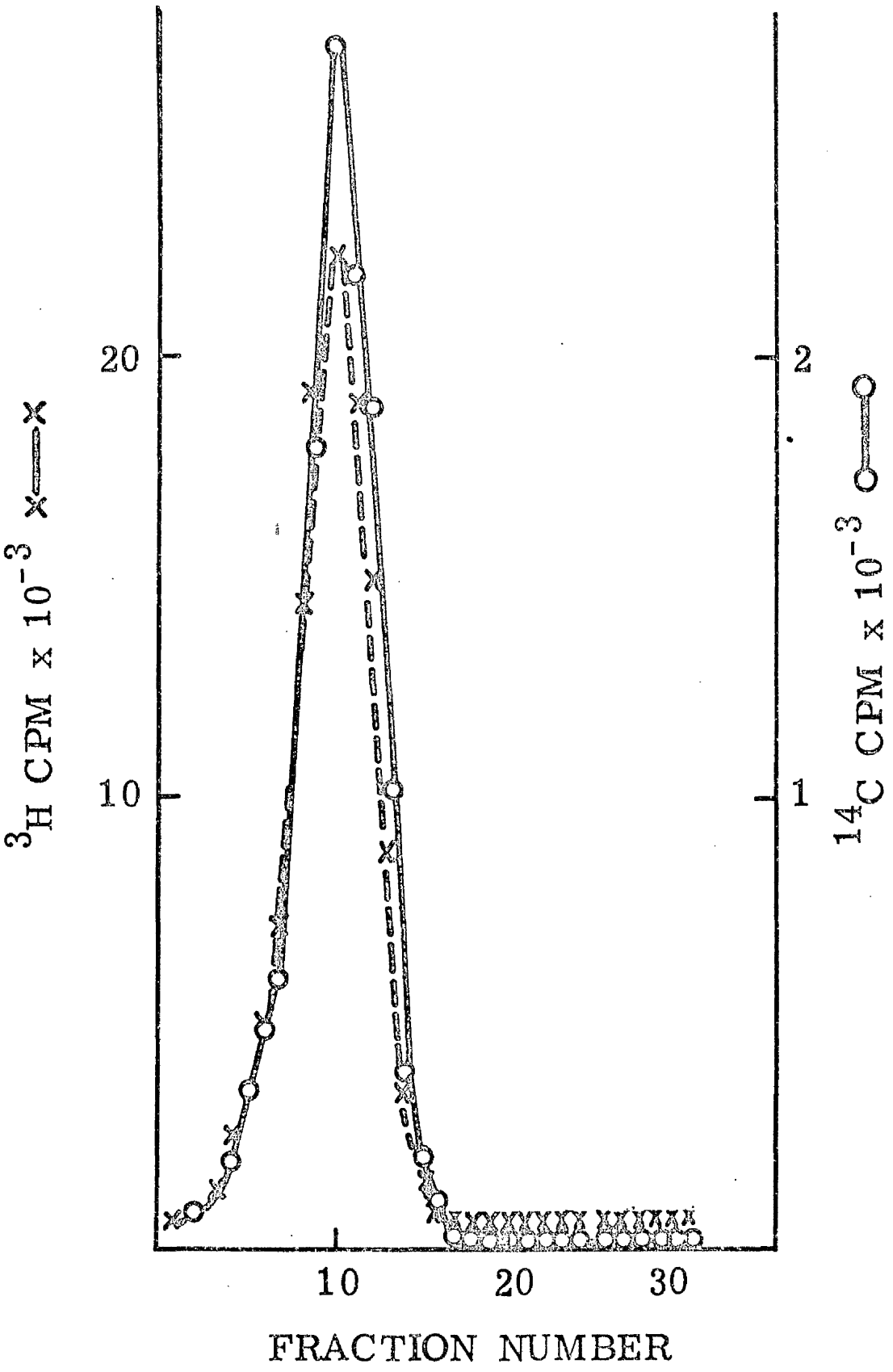
that only negligible amounts of 40S material were present.

Extraction of cultures with triton X 100 demonstrated that the incorporation of ^{3}H-leucine into 40S component was unaffected by concentrations of actinomycin D as high as 0.4 µg/ml. However, the nuclear 40S component is sensitive to the action of the drug. Presumably, newly-synthesised proteins become associated with preformed ribonucleoproteins in the cytoplasm to produce a labelled 40S component.

However, in nuclear extracts, newly-synthesised proteins become associated only with newly-synthesised RNA, the synthesis of which is sensitive to actinomycin D. The pool of pre-synthesised ribonucleoprotein within the nucleus at any given time is probably very small. The nuclear extraction procedure, coupled with the use of actinomycin D therefore eliminates incorporation of ^{3}H-leucine into the 40S ribonucleoprotein component.

The effect of 0.1 µg/ml actinomycin D on the production of SV40 DNA-protein complexes was initially studied in Triton extracts. SV40 virus-infected monolayer cultures of BSC-1 cells were pretreated with actinomycin D then labelled 3 hr with ^{3}H-thymidine. The resulting nucleoprotein complex was observed to have sedimentation characteristics idemtical to those of ^{14}C-labelled complex prepared in the absence of actinomycin D (Fig. 10). Further experiments demonstrated that 0.1 µg/ml actinomycin D did not quantitatively

Fig.10. Sedimentation analysis of SV40 nucleoprotein complex synthesised in the presence of actinomycin D. 2 resting monolayer cultures of BSC-1 cells were infected with SV40 virus (100 pfu/cell). After 27 hr, the medium from each culture was removed and replaced with Eagle's medium containing actinomycin D (0.1 µg/ml) or no actinomycin D. After a 1 hr incubation period, ^{3}H-thymidine was added to the drug-treated culture to a final concentration of 5 µCi/ml whereas ^{14}C-thymidine was added to the non drug-treated culture to a final concentration of 1.25 µCi/ml. The cultures were exposedto isotope for 3 hr prior to extraction with triton X-100. 0.1 ml aliquots of the 2 triton extracts were then mixed prior to centrifugation through a 5-20% sucrose gradient in the Spinco SW56Ti rotor at 55,000 rev/min at 4 C for 50 min.


20
10
2
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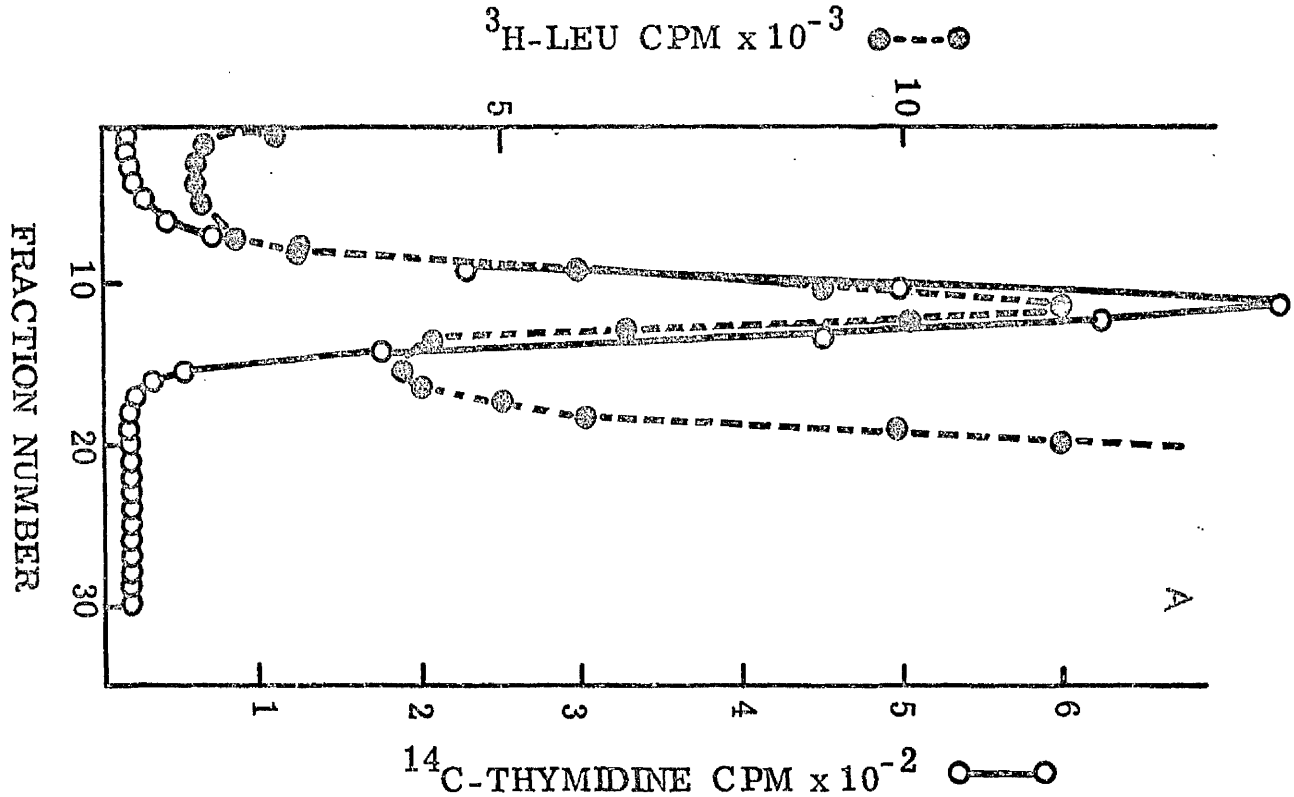
30
^{3}H CPM x 10^{-3} x—x
^{14}C CPM x 10^{-3} o—o
FRACTION NUMBER

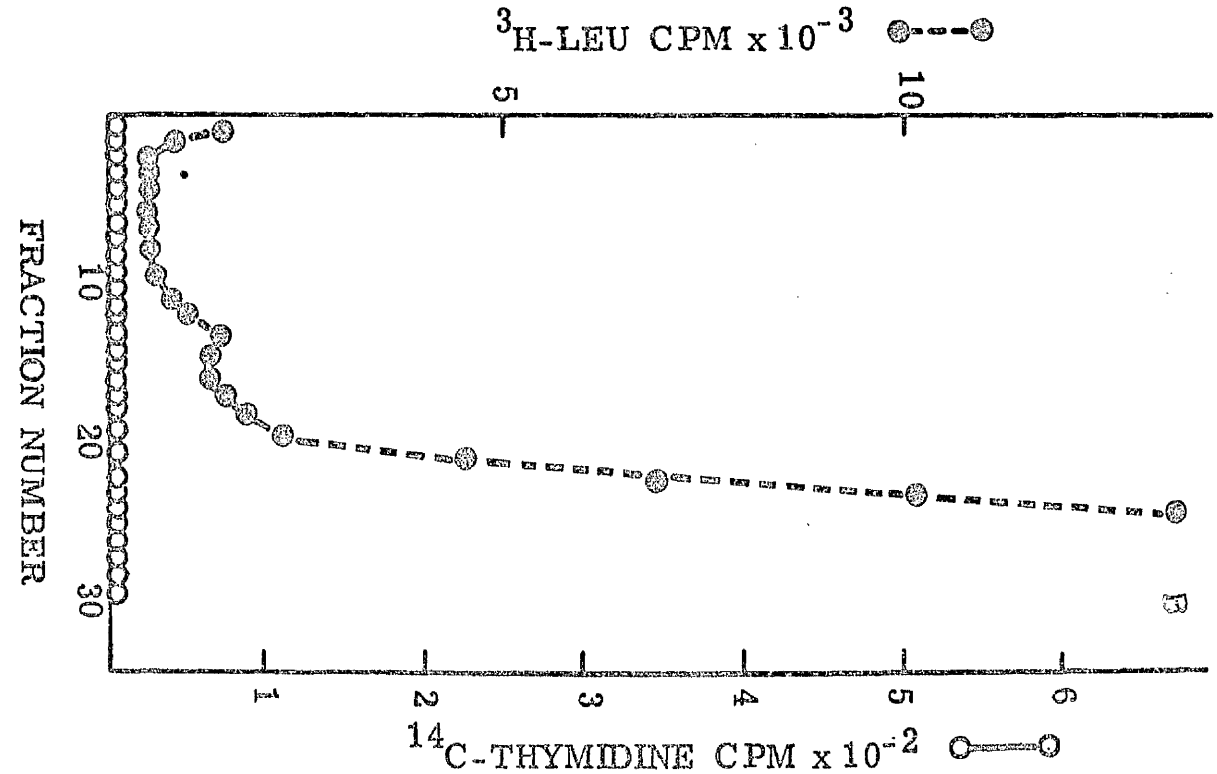
affect synthesis of the complex under the conditions employed.

The incorporation of labelled amino acids into the proteins of SV40 DNA-protein complexes was then studied in the presence of actinomycin D by means of the nuclear extraction procedure. Infected cells, 27 hr post infection, were treated with actinomycin D for 1 hr then labelled with ^{3}H-leucine and ^{14}C-thymidine in leucine-free Eagle's medium for 3 hr. Analysis of the nuclear extract on sucrose gradients (Fig. 11A) demonstrated that a large amount of material labelled with ^{3}H-leucine cosedimented with the ^{14}C-labelled complex. It is possible, however, that infection with SV40 virus might stimulate synthesis of 40S component so as to alter the sensitivity to the level of actinomycin D used above. To investigate this possibility, it was necessary to study the actinomycin D sensitivity of ^{3}H-leucine incorporation into the nuclear 40S component in SV40-infected cells in which the synthesis of the 44S viral nucleoprotein complex had been suppressed and in which, therefore, selective incorporation of ^{3}H-leucine into the 40S component could be achieved. Arabinofuranosylcytosine (ara-C) is a potent inhibitor of DNA synthesis. In order to determine precisely its effect on SV40 DNA synthesis, 3 monolayer cultures of SV40-infected BSC-1 cells were treated with 28 µg/ml ara-C at 28 hrs post infection and were pulsed with ^{3}H-thymidine at varying times thereafter for

Fig.11. Sedimentation analysis of nuclear extracts from SV40 infected BSC-1 cells.

Two resting cultures of BSC-1 cells were infected with SV40 virus (100 pfu/cell). After 27 hr, the medium was removed from each culture and replaced with warm Eagle's medium containing actinomycin D and further incubated. 30 min later, arabinocytosinylfuranoside (28 µg/ml) was added to one of the cultures (B). After a further 30 min incubation, the medium from both cultures was removed and replaced with 4 ml of warm leucine-free Eagle's medium containing actinomycin D (0.1 µg/ml), ^{3}H-leucine (50 µCi/ml), ^{14}C-thymidine (1.25 µCi/ml), and arabinocytosinylfuranoside (28 µg/ml) (B) or no ara-C (A). After a 3hr exposure to isotope, nuclear extracts were prepared and centrifuged for 3.5 hr through 5-20% sucrose gradients in the Spinco SW40Ti rotor at 40,000 rev/min in a Beckman L2-65B ultracentrifuge at 4 C. Fractions were collected and 50 µl aliquots of each were assayed for radioactivity.


^{3}H-LEU CPM x 10^{-3}
5
10
FRACTION NUMBER
10
20
30
A
1
2
3
4
5
6
^{14}C-THYMIDINE CPM x 10^{-2}


^{3}H-LEU CPM x 10^{-3}
5
10
FRACTION NUMBER
10
20
30
B
1
2
3
4
5
6
^{14}C-THYMIDINE CPM x 10^{-2}

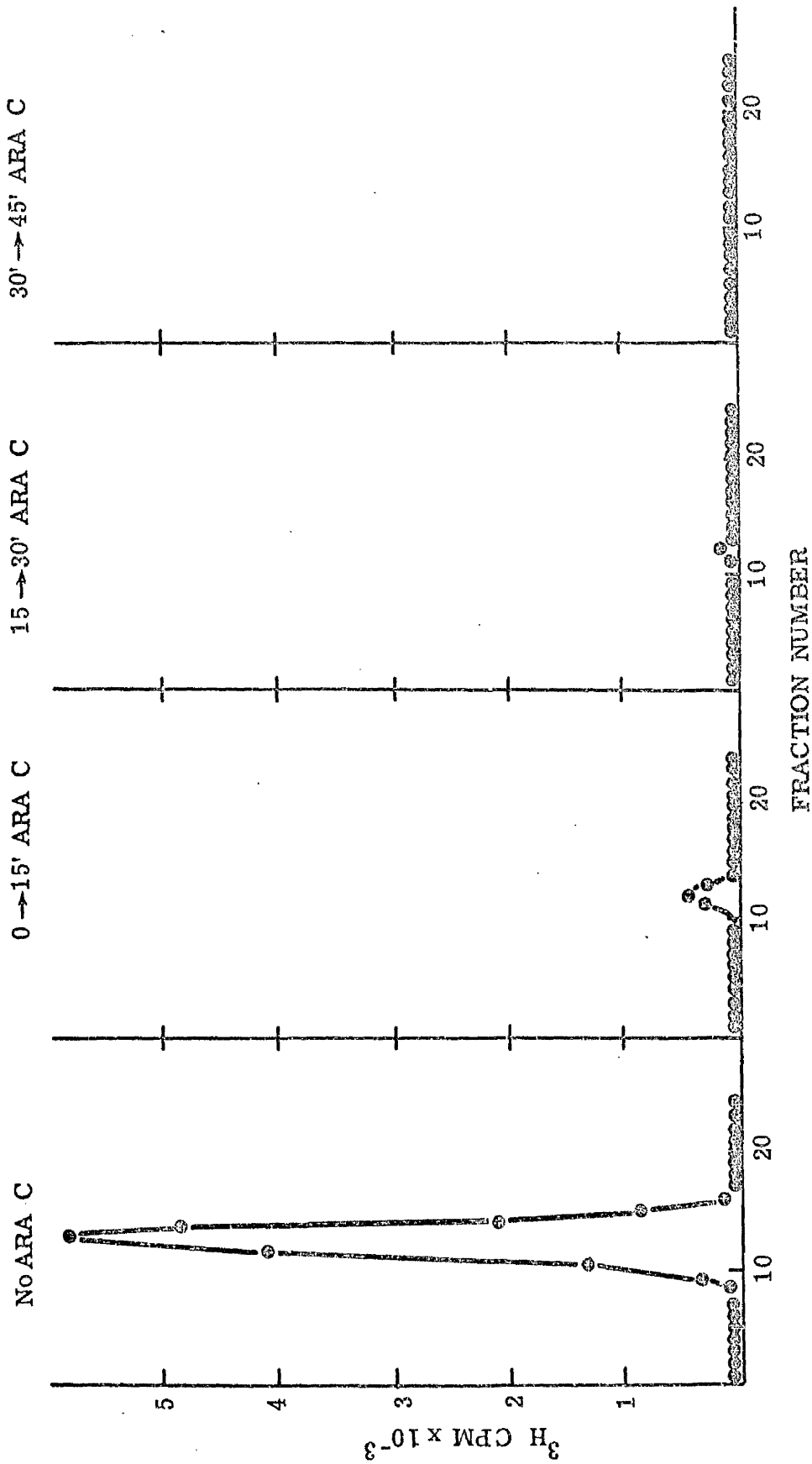
15 min periods. In each case, the pulse was terminated by extraction of the cultures with Triton. Samples of each extract were analysed by CsCl velocity sedimentation and the ^{3}H-radioactivity incorporated into 21S SV40 DNA in each case was compared to that obtained on analysis of an extract made from a parallel infected culture labelled with ^{3}H-thymidine in the absence of ara-C (Fig. 12). Ara-C was observed to completely inhibit SV40 DNA synthesis after 30 min of treatment.

Since ara-C has no effect on incorporation of ^{3}H-leucine into the 40S component in uninfected cells, this drug was used to supress synthesis of SV40 nucleoprotein complex so that incorporation of ^{3}H-leucine into the 40S component could be evaluated. BSC-1 cells were treated as in Fig. 11A except that 28 µg/ml ara-C was added 30 min prior to addition of ^{3}H-leucine. Sucrose gradient analysis (Fig. 11B) demonstrated total inhibition of SV40 nucleoprotein complex and the level of ^{3}H-leucine incorporation into 40S material was similar to those observed in uninfected cells in the presence of 0.1 µg/ml actinomycin D (c.f. Fig. 9C).

It is concluded from these results that the SV40 DNA-protein complex has been isolated in a state of radiochemical purity and is essentially free of extraneous labelled proteins.

Fig.12. Effect of ara-C on SV40 DNA synthesis.

At 28 hr post-infection with SV40 virus, the medium was removed from 3 monolayer cultures of BSC-1 cells and replaced with warm Eagle's medium containing ara-C (28 µg/ml). At 0 min (B), 15 min (C), and 30 min (D) thereafter, ^{3}H-thymidine was added to a final concentration of 5 µCi/ml and, in each case, the cultures were exposed to the isotope for 15 min and subsequently extracted with triton X-100. A parallel SV40 infected culture was similarly labelled with ^{3}H-thymidine in the absence of ara-C at 28 hr post-infection and a triton extract was prepared (A). 0.2 ml samples of each of the above extracts were analysed by velocity centrifugation through neutral CsCl (density 1.50 g/ml) in the Spinco SW50.1 rotor at 40,000 rev/min for 3 hr at 20 C.


No ARA C
0 → 15' ARA C
15 → 30' ARA C
30' → 45' ARA C
^{3}H CPM x 10^{-3}
1
2
3
4
5
10
20
FRACTION NUMBER

Characterisation of polypeptides in the nucleoprotein complex.

SV40 virus-infected BSC-1 cells were exposed to ^{3}H-leucine in the presence of actinomycin D as before and nucleoprotein complex was isolated by sucrose gradient centrifugation of nuclear extracts. The ^{3}H-labelled 44S complex was mixed with purified SV40 virus, which had been labelled with ^{14}C-protein hydrolysate, and the proteins in the mixture were disaggregated in SDS, ME and 8M urea prior to electrophoresis in SDS-polyacrylamide gels (Fig. 13). At least seven discrete components, including all of those present in the mature virion were observed in the nucleoprotein complex. It has not proved possible to resolve clearly the 3 virus proteins VP4, VP5 and VP6 (Barban & Goor, 1971). The complex is particularly rich in the small polypeptides VP4-6. VP1 was also observed in the complex but the radioactivity in this component was only 7% of the total radioactivity compared with 75% in the mature virus. Material with a molecular weight of 80,000 was clearly evident, especially in the nucleoprotein complex. A component was observed (M.W. 28,000) which did not comigrate with any of the ^{14}C-viral marker proteins. Material from experiments shown in Fig. 11B gave no discrete peaks in SDS gels. The percentage of radioactivity in the proteins in ^{3}H-leucine-labelled complex has also been compared to those in both SV40 virus and empty shells labelled with

Fig.13. Acrylamide gel electrophoresis of the protein components of SV40 nucleoprotein complex.

^{3}H-leucine labelled SV40 nucleoprotein complex was isolated as described in Fig.11 (A) and the peak fractions in the 44S region (fractions 9,10,11 in Fig.11 (A)) were pooled. A 0.1 ml aliquot of this pool was mixed with purified ^{14}C-amino acid-labelled SV40 virus to provide markers for the viral coat proteins. After disruption, the sample was analysed by electrophoresis on a 10% acrylamide gel containing 0.1% SDS as described in the Materials and Methods section.

A pool was made from the corresponding fractions from the ara-C treated culture described in Fig.11 (B) and was similarly analysed by SDS acrylamide gel electrophoresis (^{3}H cpm x----x)

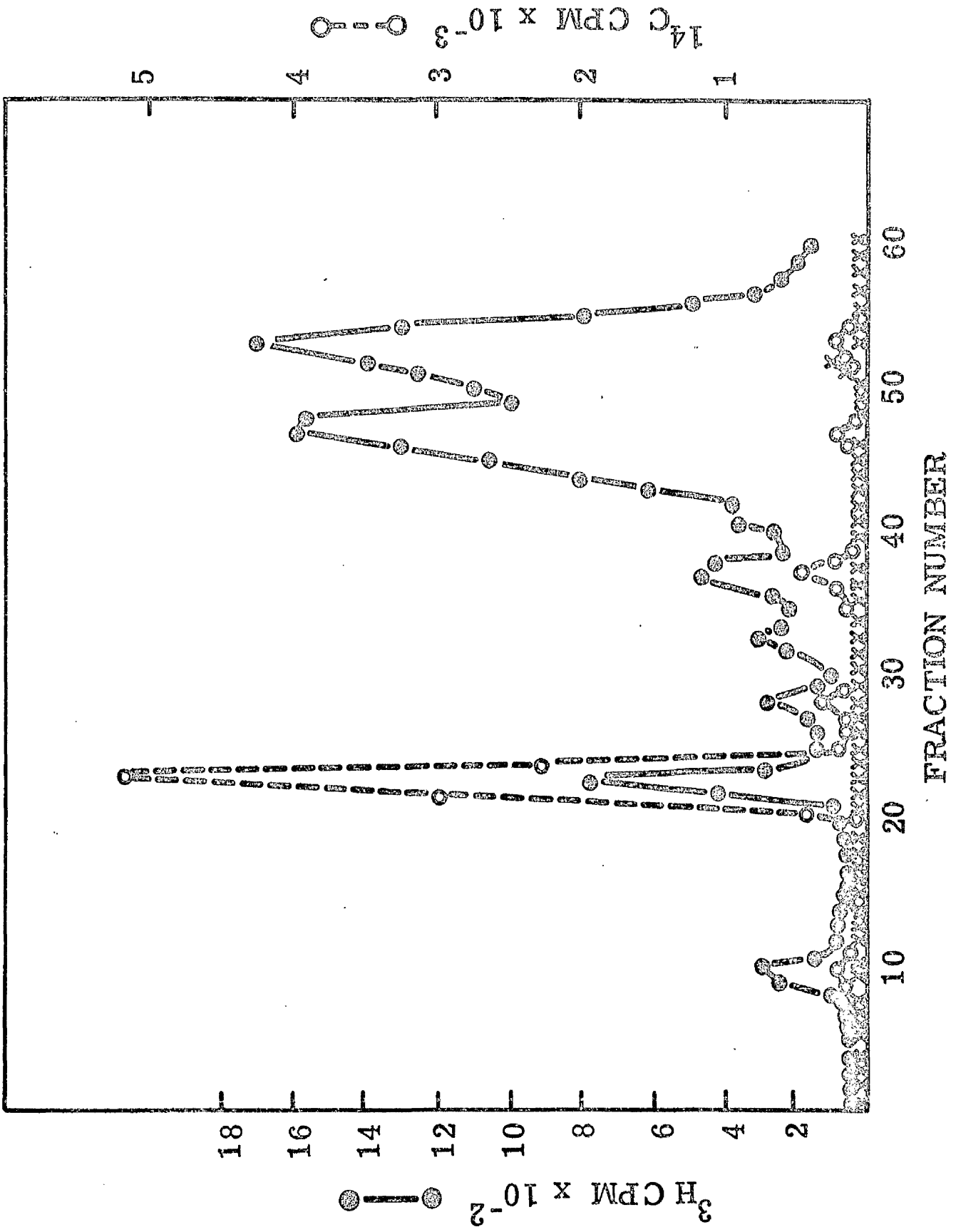

^{14}C CPM x 10^{-3}
5
4
3
2
1
FRACTION NUMBER
10
20
30
40
50
60
^{3}H CPM x 10^{-2}
18
16
14
12
10
8
6
4
2

Fig.14. Acrylamide gel electriphoresis of the protein components of 'full' SV40 virus and SV40 'empty' shells. Two monolayer cultures of BSC-1 cells were infected with 0.01 pfu/cell of SV40 virus and, 80 hr later, were labelled with ^{3}H-lysine (200 µCi/dish). The cells were harvested 80 hr later and 'full' SV40 virus and 'empty' SV40 shells were purified as described as in the Materials & Methods section.

After disruption, a 50 µl aliquot of 'full' virus (A) or 'empty' shells (B) were analysed by electrophoresis on a 10% acrylamide gel containing 0.1% SDS as described in the Materials & Methods section.

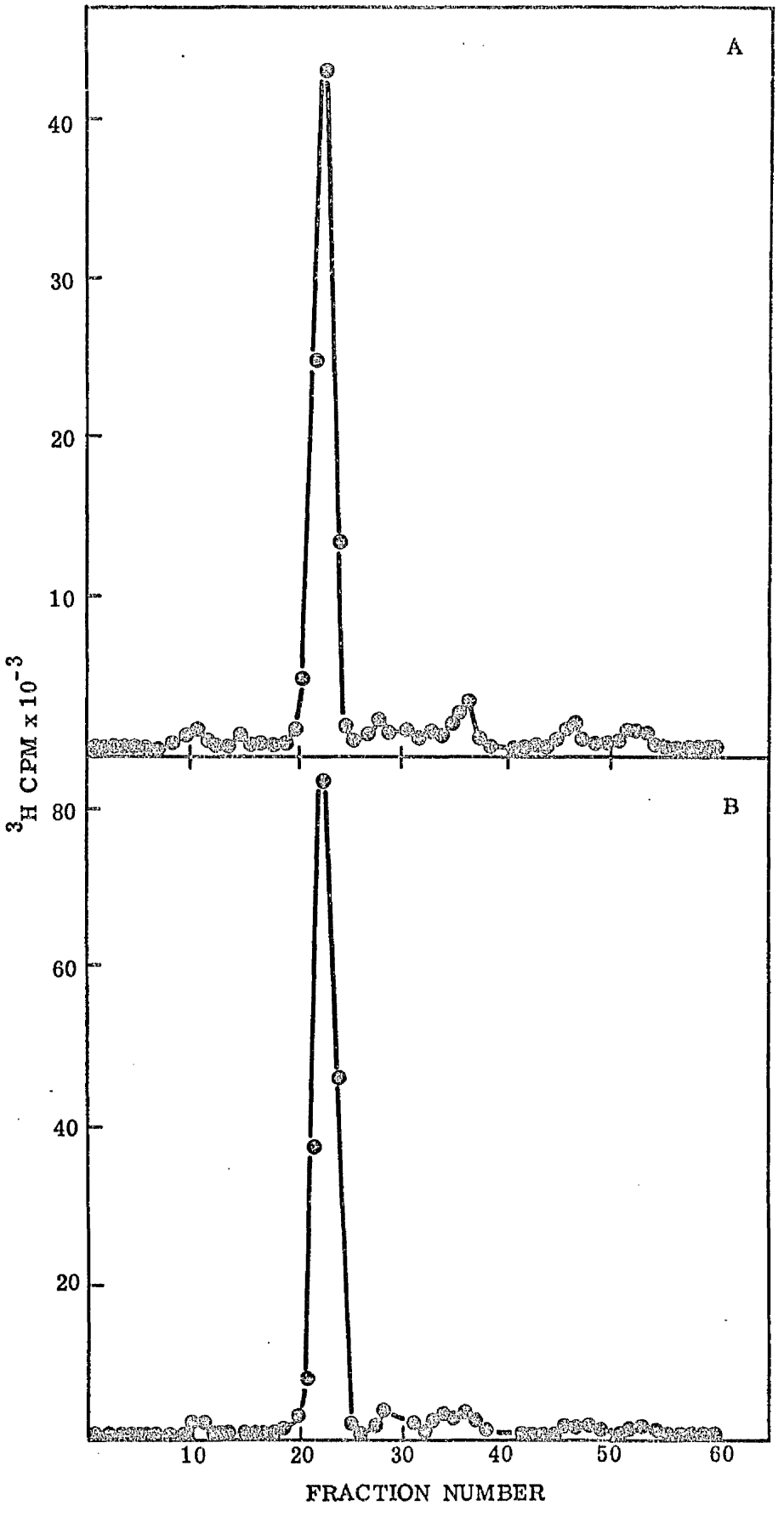

A
B
40
30
20
10
80
60
40
20
^{3}H CPM x 10^{-3}
10
20
30
40
50
60
FRACTION NUMBER

TABLE 1

Distribution of leucine in purified viral components

	% of total in constituent proteins			
Preparation	VP1	VP2	VP3	VP4-6
SV40 virus	75	4	8	11
Empty shells	77	6	8	4.
Complex	7	1	6	82

Purified SV40 virus, SV40 'empty' particles and SV40 nucleoprotein complex (all labelled with ^{3}H-leucine) were analysed by SDS-polyacrylamide gel electrophoresis and the amount of radioactivity in each of the major viral components was measured and expressed as a percentage of the total radioactivity recovered from the gel.

^{3}H-leucine (Table 1).

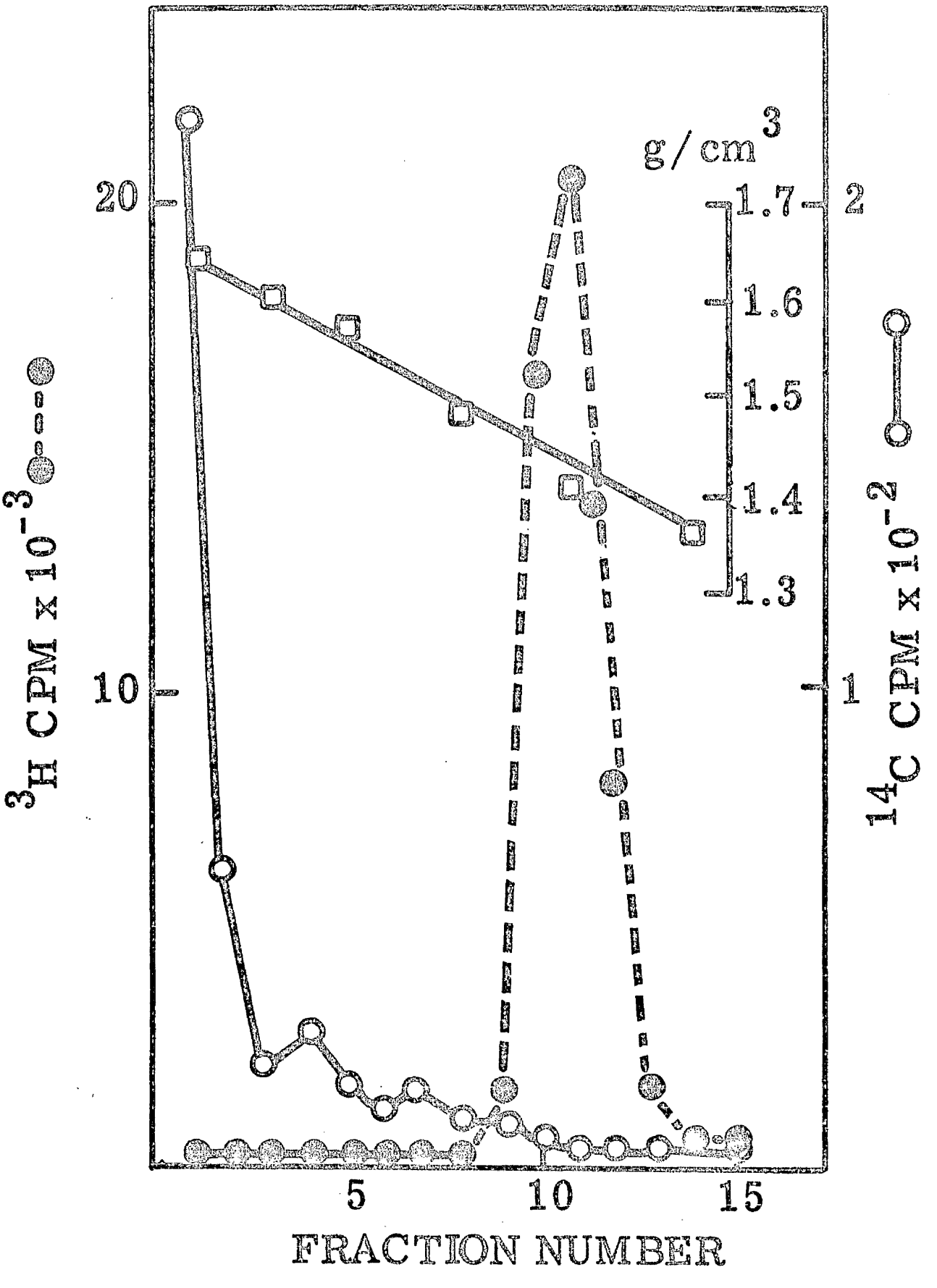
The total counts in the region VP4-6 have been combined. The ^{3}H-radioactivity profiles obtained on SDS-polyacrylamide gel analysis of disrupted SV40 virus (A) and SV40 'empty' shells (B) are shown in Fig. 14 and are qualitatively similar to the profiles obtained with ^{3}H-leucine labelled particles. The 'empty' shells have a reduced content of VP4-6 compared with full virus (Fig. 14 & Table 1).

Protein-DNA ratio in the complex.

It is clear that an unambiguous determination of the protein-DNA ratio in SV40 DNA-protein complexes requires a complete chemical analysis of the constituent proteins. Since it has not yet been possible to achieve chemical purification, as opposed to radiochemical purification, we have obtained a preliminary estimate on the basis of the buoyant properties of complex fixed with glutaraldehyde. The principal objection to this approach is that any contaminating unlabelled proteins might bind to the complex under these conditions. In an effort to determine the extent of random attachment of protein, ^{14}C-labelled 21S SV40 DNA was mixed with Triton-extracted nucleoprotein complex, which had been labelled with ^{3}H-thymidine, prior to glutaraldehyde treatment. Buoyant analysis in CsCl gradients (Fig. 15) demonstrated that the buoyant density of the complex was 1.42 g/ml. The ^{14}C-DNA was found at

Fig.15. Buoyant properties of SV40 nucleoprotein complex after fixing with glutaraldehyde.

BSC-1 cells were infected with SV40 virus (100 pfu/cell) and were labelled with 5 μCi/ml of ^{3}H-thymidine for 3 hr prior to extraction with triton X-100. 0.15 ml of this extract was mixed with 50 μl of ^{14}C-labelled 21S SV40 DNA and the mixture was subsequently made 1% with respect to glutaraldehyde. After 15 min at 20 C, 0.1 ml of sample was added to 3 ml of CsCl (density 1.50 g/ml) and centrifuged for 40 hr in the Spinco SW50.1 rotor at 40,000 rev/min at 20 C.


g/cm 3
1.7
1.6
1.5
1.4
1.3
20
10
2
1
^{3}H CPM x 10^{-3}
^{14}C CPM x 10^{-2}
5
10
15
FRACTION NUMBER

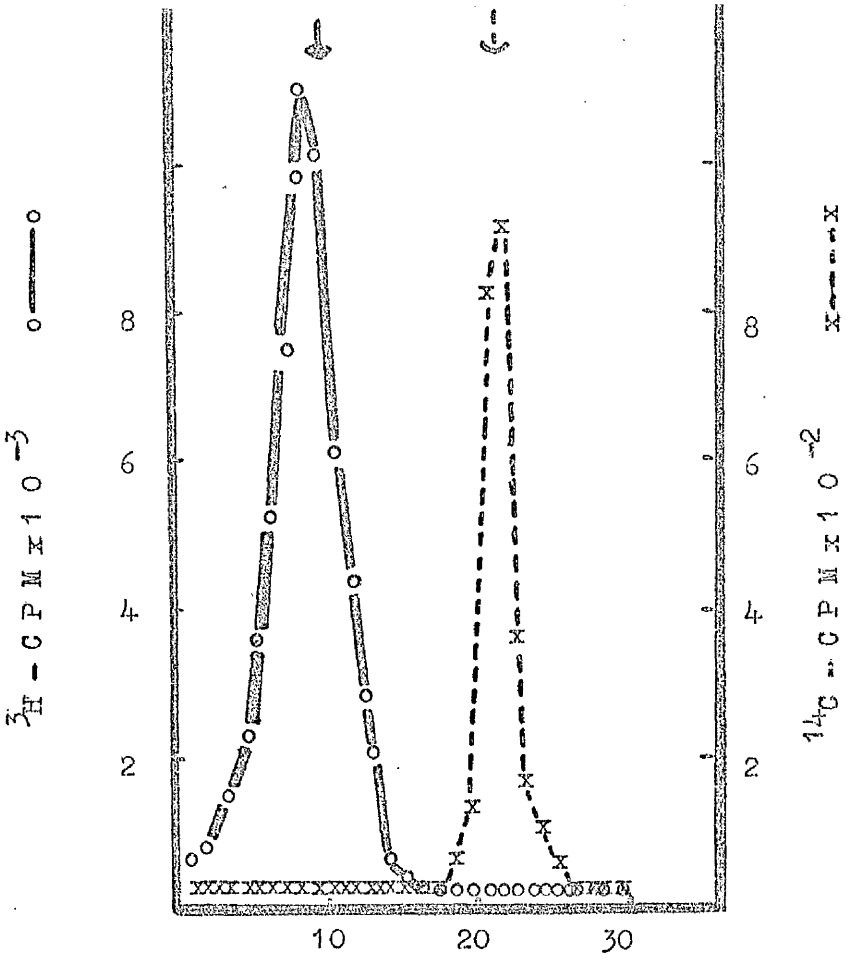


Fig. 16. Sedimentation properties of SV40 nucleoprotein complex after fixing with glutaraldehyde.

A 0.1 ml aliquot of the glutaraldehyde treated material described in Fig.15 was centrifuged through a 5-20% sucrose gradient at 55,000 rev/min for 50 min in the Spinco SW56Ti rotor at 4 C. The arrows mark the positions in the gradient of non glutaraldehyde-treated 44S complex (⇩) and 21S SV40 DNA (⇩).

the bottom of the tube. Further experiments (Fig. 16) demonstrated that the sedimentation coefficient of the complex did not fall below 44S, indicating that no disruption of the complex was produced by the glutaraldehyde treatment. In addition, the sedimentation coefficient of the 21S ^{14}C-SV40 DNA marker was unaltered, so that non-specific attachment of proteins to the DNA had not occurred to a detectable extent. Calculation on a weight percent basis indicated that the percentage of protein in SV40 DNA-protein complexes is of the order of 60% and the molecular weight of the complex is therefore in the range $7.5 - 8.5 \times 10^6$.

Replicating SV40 DNA can be Detected as a Nucleoprotein Complex.

Levine et al (1970) demonstrated that when SV40-infected AGMK cells were exposed to ^{3}H-thymidine for periods of the order of 5 min, little or no radio-activity was present as 21S SV40 DNA. Instead, a component, which had the properties of a replicative intermediate DNA molecule, was present and had a sedimentation coefficient of 25S.

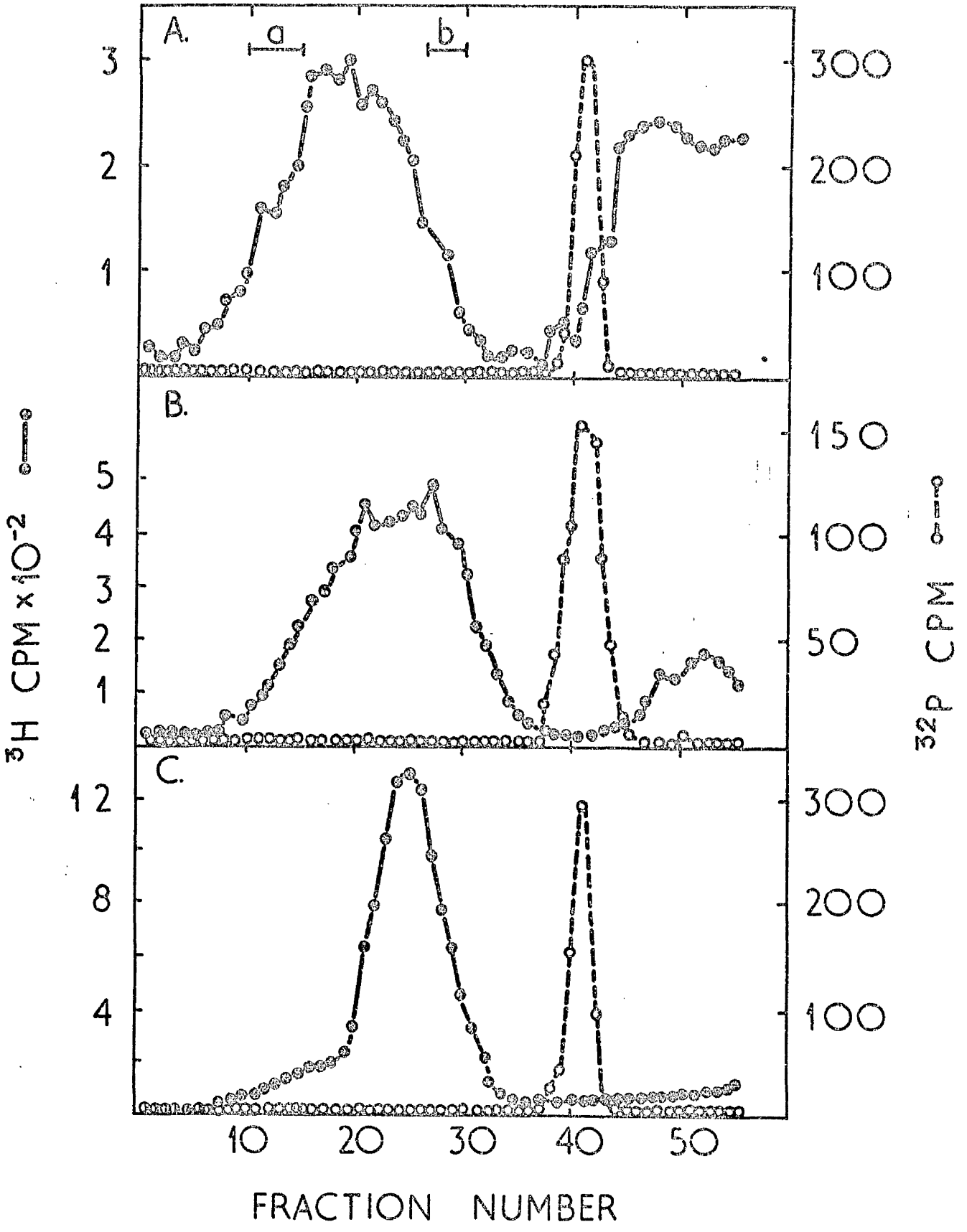
In view of these results, short term labelling experiments were performed with SV40-infected BSC-1 cells to determine the nature of such replicating DNA molecules in triton extracts. Six monolayer cultures of BSC-1 cells were infected with SV40 virus and 30 hr post-infection, the medium was removed and replaced with 2 ml Eagle's medium containing ^{3}H-thymidine (80 μCi/ml) in the absence

of serum. After 5 min incubation at 37 C, the radioactive medium was removed. The cells were then washed with PBS, and four of the dishes were overlayed with 10 ml Eagle's medium containing unlabelled thymidine (500 µg/ml) and incubated at 37 C. The fifth dish was extracted with 0.25% triton, whilst the sixth was extracted with 0.25% deoxycholate, which extracts intracellular pools of unencapsulated SV40 DNA (Bourgaux et al 1969; Eason & Vinograd, 1971). After a 5 min chase, the first two dishes were extracted, one with triton and the other with deoxycholate as described previously. Similarly, after a 15 min chase, the remaining dishes were treated as in the case of the 5 min chase.

Samples (0.2 ml) of the three triton extracts were analysed by sucrose gradient centrifugation to detect the presence of nucleoprotein complexes and by velocity analysis on CsCl to characterise the DNA species present. The deoxycholate extracts were also analysed by CsCl velocity centrifugation. Analysis of the triton extracts on sucrose gradients is shown in Fig. 17.

In cells which had been labelled with ^{3}H-thymidine for 5 min, the triton extract contained material with a broad sedimentation distribution profile (45S-100S) (Fig. 17A). Samples in the regions indicated by the horizontal bars were pooled to determine the type

Fig.17. Sedimentation of triton extracts from SV40-infected BSC-1 cells exposed to ^{3}H-thymidine then chased with cold thymidine. Infected cells 30 hr post-infection were labelled for 5 min (A) then chased with cold thymidine for 5 min (B) and for 15 min (C). ^{32}P-labelled 21S SV40 DNA was added as marker and samples were centrifuged in 5-20% sucrose gradients for 2 hr in the SW65L rotor at 36,000 rev/min at 4C in the Beckman L2-65B ultracentrifuge. The horizontal bars in (A) represent fractions pooled for subsequent analysis of the DNA species present in these regions of the distribution.

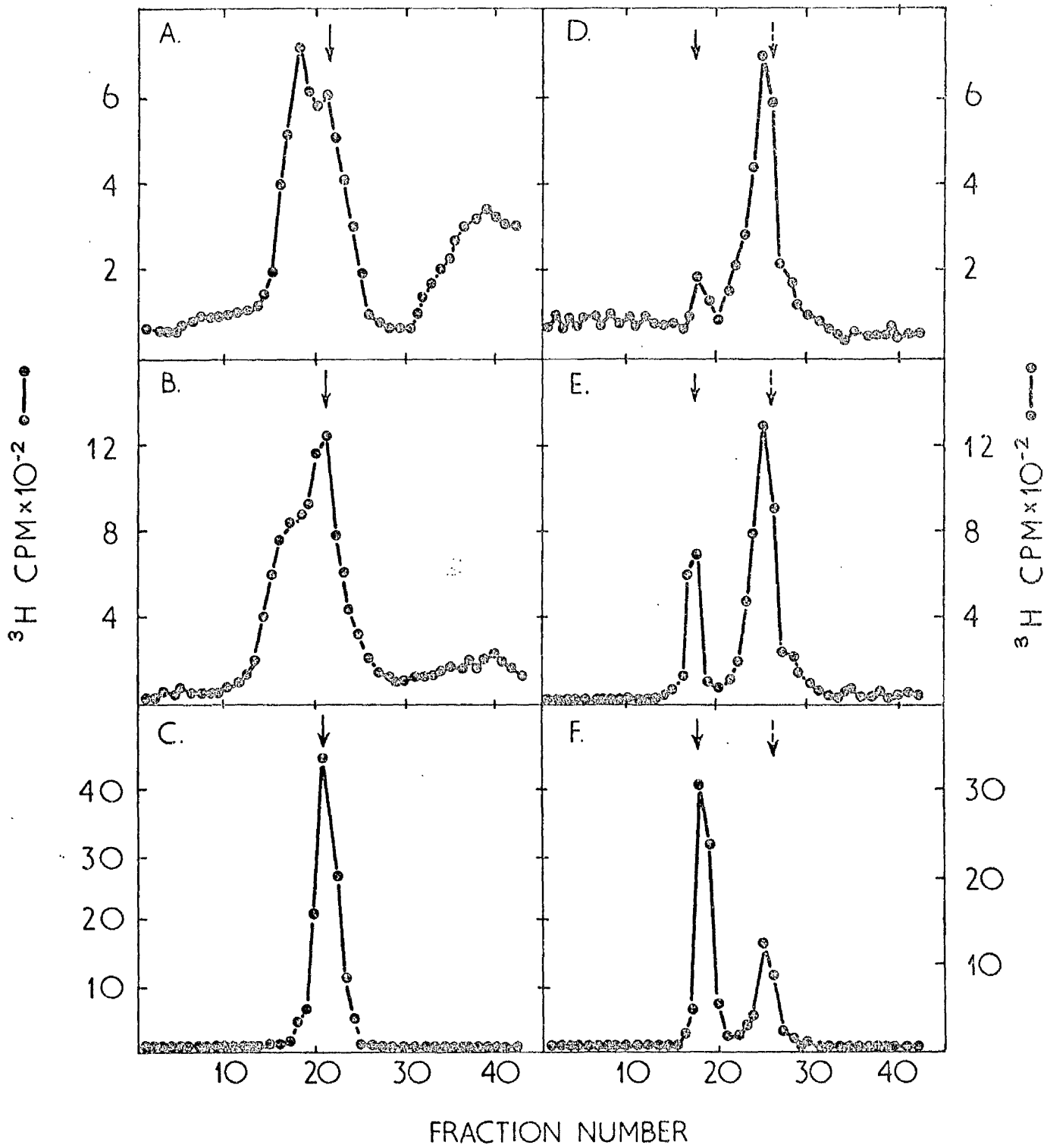

A.
a
b
B.
C.
^{3}H CPM x 10^{-2}
^{32}P CPM
FRACTION NUMBER

of DNA present in these parts of the gradient. In triton extracts obtained after a 5 min chase period (Fig. 17B), the proportion of material with high S values was diminished, but a significant amount sedimented faster than the 44S component in the form of a diffuse leading boundary. After a 15 min chase period (Fig. 17C), the triton extract contained a population of molecules which appeared to be much less polydisperse and which consisted mainly of 44S material. These results indicate progressive conversion of rapidly-sedimenting material to the 44S component. Material with sedimentation properties of 25S or 21S SV40 DNA was not observed in any significant amount when the triton extracts were analysed on sucrose gradients. It is therefore possible that the intracellular pools of SV40 DNA may not occur in the free state but are associated with protein.

The properties of DNA present in the triton extracts were analysed by CsCl velocity centrifugation and by means of CsCl-PDI gradients at equilibrium (Fig. 18).

These results demonstrate that, after a five min exposure to ^{3}H-thymidine, a large proportion of the DNA in the triton extract was in the form of 25S SV40 DNA (Fig. 18A). The 25S DNA was progressively chased into 21S SV40 DNA (Figs. 18B, 18C). An essentially similar sequence of events was observed to occur in the SV40 DNA extracted with deoxycholate in agreement with the

Fig.18. Analysis of triton extracts by CsCl velocity centrifugation and CsCl-PDI buoyant centrifugation after exposure to ^{3}H-thymidine followed by chase with cold thymidine. Three dishes of cells, 30 hr post-infection with SV40 virus, were labelled with ^{3}H-thymidine for 5 min. One dish was extractedwith triton for velocity analysis (A) and buoyant analysis (D). The remaining dishes were washed free of isotope and medium containing cold thymidine was added. After 5 min chase, one dish was extracted for velocity(B) and buoyant (E) analysis. After 15 min chase, the remaining dish was extracted with triton for velocity (C) and buoyant (F) analysis. For velocity centrifugation, samples were mixed with ^{32}P-labelled 21S SV40 DNA and layered on 3 ml CsCl (density 1.50 g/ml). Centrifugation was performed for 3 hr in a SW50L rotor at 40,000 rev/min at 20 C. The arrow indicates the position of 21S marker DNA. For buoyant analysis in CsCl-PDI gradients, samples were mixed with ^{32}P-labelled 21S (form 1) and 16 S (form 2) SV40 DNA and centrifuged in CsCl (density 1.52 g/ml)-PDI (500 µg/ml) for 40 hr in a Spinco SW50L rotor at 40,000 rev/min at 20 C. The arrows indicate the positions of the marker DNA species.


A.
B.
C.
D.
E.
F.
6
4
2
12
8
4
40
30
20
10
10
20
30
40
^{3}H CPM x 10^{-2}
FRACTION NUMBER

observations of Levine et al (1970). However, the small molecular weight DNA observed in short labelling and chase periods in these experiments with BSC-1 cells (Fig. 18A and Fig. 17A) were not reported to occur in SV40-infected AGMK cells (Levine et al 1970). We cannot at present exclude the possibility that this small DNA chases into 21S viral DNA. The small DNA may alternatively arise from the host cell, since this material is also observed in uninfected cells labelled for short periods with ^{3}H-thymidine.

Analysis of the samples by means of CsCl-PDI gradients at equilibrium, demonstrated clearly the progressive conversion of 25S DNA to 21S DNA with increasing time of chase with cold thymidine (Figs. 18D, 18E and 18F). It was observed in Fig. 18D that the peak of replicating SV40 DNA was present as a broad heterogeneous distribution which extended to the 21S DNA marker position in a manner similar to that reported by Sebring and coworkers (1971) and by Jaenisch & Levine (1971). The distribution became more clearly resolved after 5 and 15 min chase with cold thymidine. The improved resolution achieved by this means allowed accurate quantitation of the percentage of 21S SV40 DNA present at each time interval and a comparison is drawn with DNA extracted from infected cells with deoxycholate at corresponding times (Table 2).

TABLE 2. Percentage of 21S SV40 DNA in triton & deoxycholate extracts.

Time of chase after 5 min exposure to ^{3}H-thymidine	Percentage of total counts/min in 21S SV40 DNA	
	Triton extract	Deoxycholate extract
0 min	14	17
5 min	26	24
15 min	71	66
55 min	100	100

These results indicate that essentially the same proportion of 21S SV40 DNA was present at corresponding times in the triton and deoxycholate extracts. Further experiments to estimate the efficiency of extraction of acid-precipitable radioactivity demonstrated that the two extraction procedures gave the same yield of DNA to within five percent. We exclude, on this basis, the possibility of selective extraction of nucleoprotein complexes with triton. That is, the DNA extracted in the form of complexes by means of triton represents at least the major portion of the DNA extractable with deoxycholate. Replicating form SV40 DNA appeared to be present only when complex was present with sedimentation coefficient greater than 44S. To confirm this finding and to test for the presence of 21S viral DNA throughout the heterogeneous distribution indicated in Fig. 17A, the regions indicated by the horizontal bars were analysed on CsCl-PDI equilibrium gradients (Fig. 19). Rapidly sedimenting material from the region indicated by bar 'a' contained only replicating form DNA (Fig. 19A) while only covalently closed SV40 DNA was present at the trailing edge (bar 'b') of the population (Fig. 19B).

In order to determine more precisely the nature of the complex containing the replicative form of SV40 DNA, a culture of BSC-1 cells was labelled with ^{14}C-thymidine for 2 hrs at 28 hr post-infection. The cells were subsequently washed free of isotope

Fig.19. Buoyant behaviour of materiat fractionated from sucrose gradients of triton extracts from SV40 infected BSC-1 cells after exposure to ^{3}H-thymidine for 5 min. Fractions were prepared from the experiment described in Fig. 17(A). Pooled samples indicated by the horizontal bars 'a' and 'b' were dialysed free of sucrose and adjusted to density of 1.52 g/ml with CsCl and to 500 µg/ml with PDI then centrifuged to equilibrium as described in the legend to Fig.18. (A) Material from the pool indicated by bar 'a' in Fig 17(A). (B) Material from the pooled samples indicated by bar 'b' in Fig. 17(A).

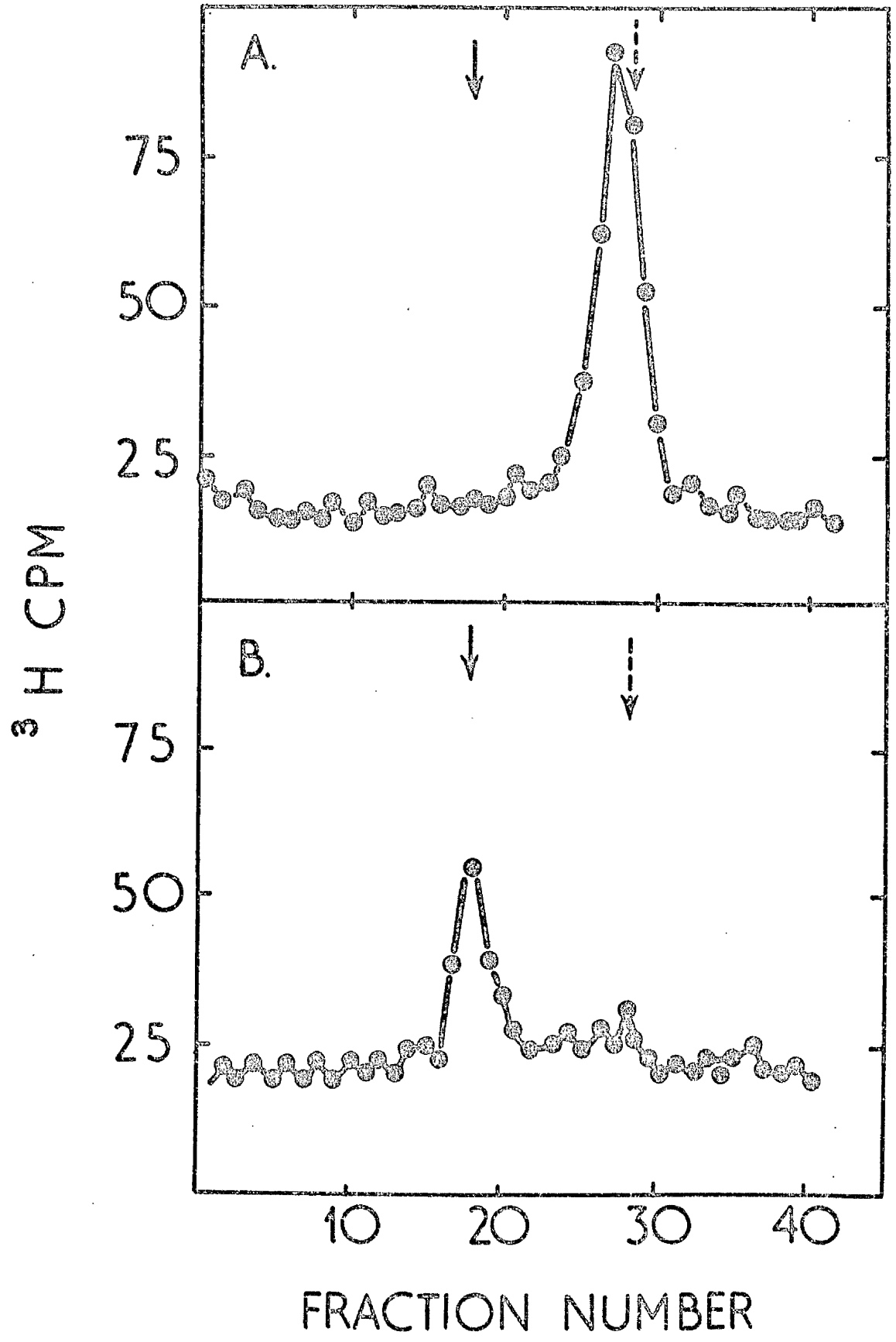
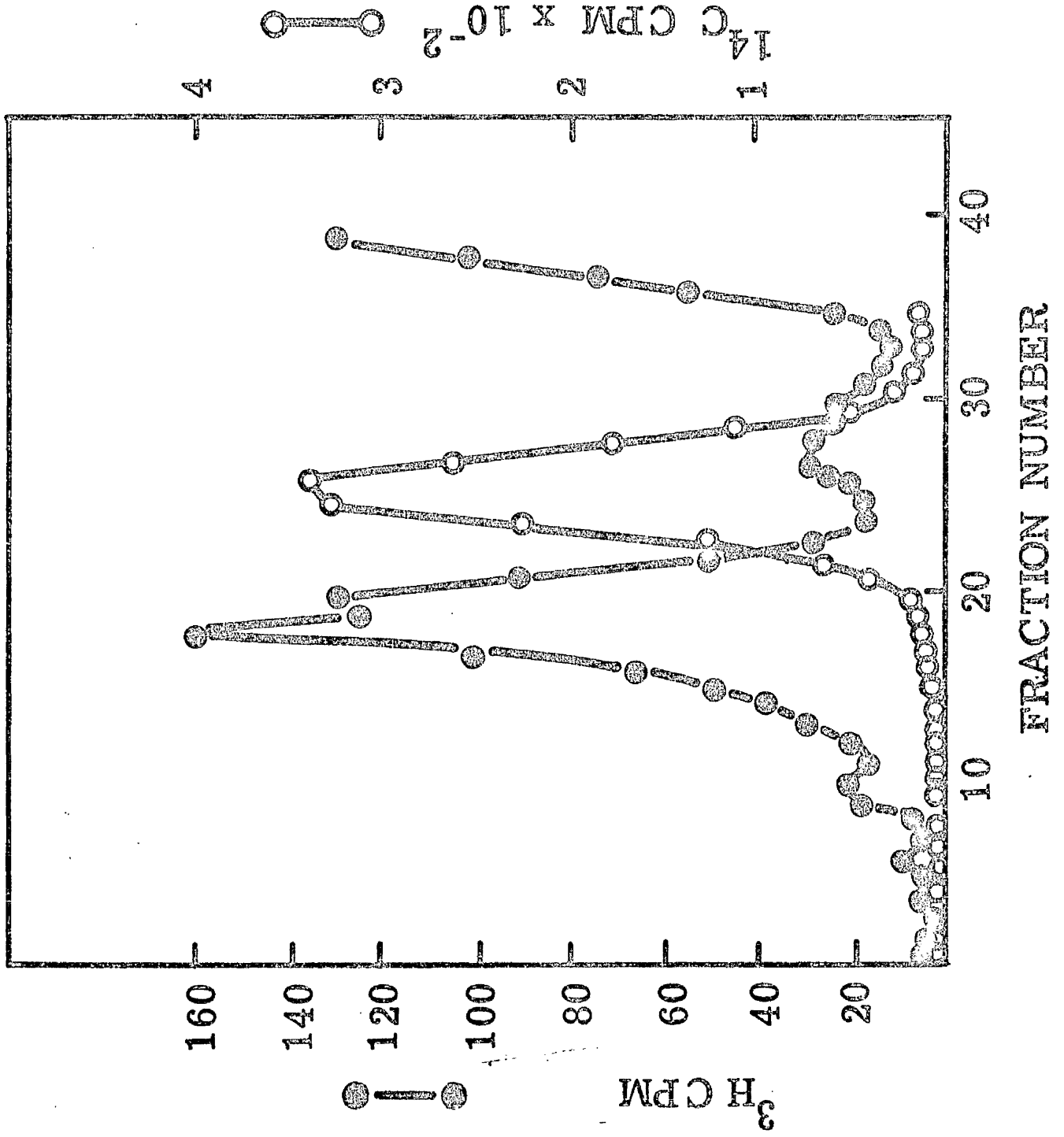

A.
B.
75
50
25
75
50
25
^{3}H CPM
10
20
30
40
FRACTION NUMBER

Fig.20. Sedimentation analysis of replicating complex. At 28 hr post-infection with SV40 virus, a monolayer culture of BSC-1 cells was exposed to ^{14}C-thymidine (1.25 μCi/ml) for 2 hr. The cells were then washed free of isotope and incubated for a further 2 hr with warm Eagle's medium. After this time, the medium was again removed and replaced with warm Eagle's medium containing ^{3}H-thymidine (100 μCi/ml). The cells were subsequently extracted with triton X-100 after a 3 min exposure to isotope and the extract thus obtained was centrifuged through a 5-20% sucrose gradient at 40,000 rev/min for 2.5 hr in the Spinco SW40Ti rotor at 4 C. The multiplicity of virus input was 20 pfu/cell


^{14}C CPM x 10^{-2}
4
3
2
1
40
30
20
10
FRACTION NUMBER
160
140
120
100
80
60
40
20
^{3}H CPM

and incubated with Eagle's medium (no serum) for a further 2 hr. After this time, the medium was removed and replaced with Eagle's medium containing ^{3}H-thymidine. The cells were extracted with triton X-100 after a 3 min exposure to isotope.

Analysis by sucrose gradient centrifugation of the triton extract revealed that the majority of the ^{3}H-labelled replicating complex sedimented around 90S compared to the ^{14}C-labelled 44S complex (Fig.20). However, in this case, conditions are such as to permit complete resolution between the replicating and mature forms of complex.

Synthesis of the complex in relation to virus growth

In order to establish the optimum time period for studying SV40 nucleoprotein complex synthesis in relation to the synthesis of virions, an experiment was performed to determine the rate of ^{3}H-thymidine incorporation into both complex and mature virus at various times after infection. The formation of infective progeny virus was also followed. At 0, 17, 29, 42, 51, 65, and 90 hr after infection with SV40, a 1hr exposure to ^{3}H-thymidine was administered to monolayer cultures of BSC-1 cells. In each case, the pulse was terminated by extraction of the cultures by triton X-100. An aliquot of each extract was centrifuged through a 5-20% sucrose gradient and the total number of counts per culture sedimenting in the 44S region

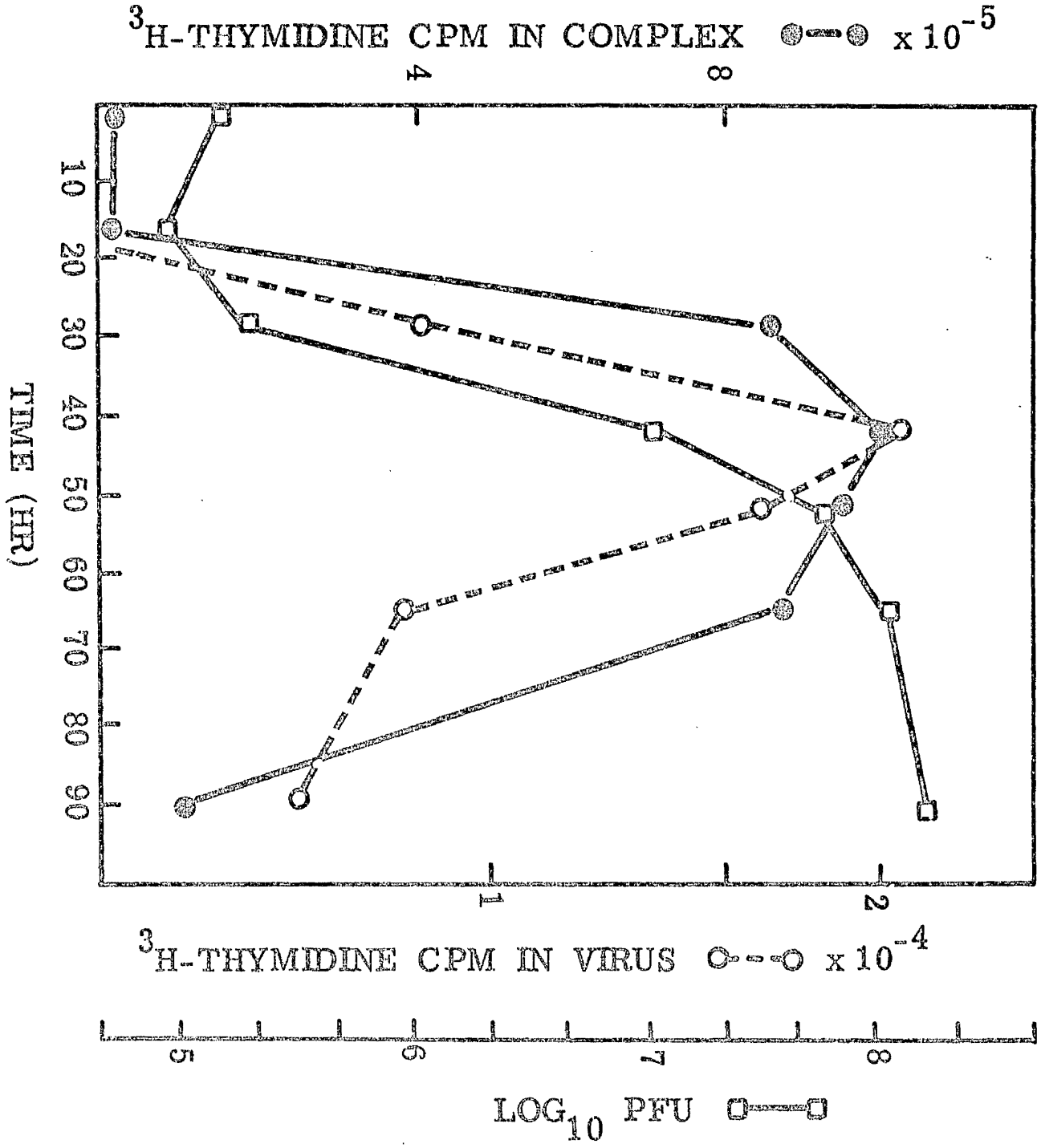
of the gradient was determined (Fig. 22). In every case, the radioactivity behaving as 44S complex accounted for all the acid precipitable radioactivity in the extract, indicating that, at all times after infection, SV40 DNA was present only as a 44S complex; complexes with altered sedimentation characteristics were not observed. ^{3}H-thymidine incorporation into 44S complex was not observed at 17 hr. post infection, but thereafter increased sharply to a maximum rate of synthesis at 42 hr. After this time, the rate of incorporation of isotope declined.

In a similar experiment, the cells were exposed to ^{3}H-thymidine for 2 hrs. at various times after infection, and were subsequently harvested and assayed both for infectious virus and for ^{3}H-radioactivity behaving as virus (see also Fig. 22).

The production of infectious virus was first noted at 29 hr post-infection and ^{3}H-thymidine incorporation reached a maximal rate of 42 hr post infection. At later times, the rate of isotope incorporation was reduced. A pronounced cytopathic effect was observed by 51 hr post infection and by 90 hrs a yield of 10^{3} pfu/cell was obtained.

Fig.22. Synthesis of the complex in relation to the synthesis of SV40 virions. At 0, 17, 29, 42, 51, 65, and 90 hr after infection with SV40 virus (input multiplicity 10 pfu/cell), ^{3}H-thymidine (20 μCi/ml) was administered to monolayer cultures of BSC-1 cells and these were exposed to the isotope for 1 hr at 37 C. In each case, the pulse was terminated by extraction of the cultures with triton X-100. A 0.2 ml aliquot of each extract was centrifuged through a 5-20% sucrose gradient in the Spinco SW50.1 rotor for 2 hr at 36,000 rev/min at 4 C and the total number of counts/min/culture sedimenting in the 44S region of the gradient was determined in each case (o —— o).

In a parallel experiment, the cells were exposed, at each of the above times post infection, to ^{3}H-thymidine (20 μCi/ml) for 2 hr and were subsequently harvested and assayed both for infectious virus (pfu/culture; □ —— □) and for ^{3}H-radioactivity in the form of virions (o - - - o) (the latter being determined as described in the legend to Fig.28 and expressed as counts/min/culture).

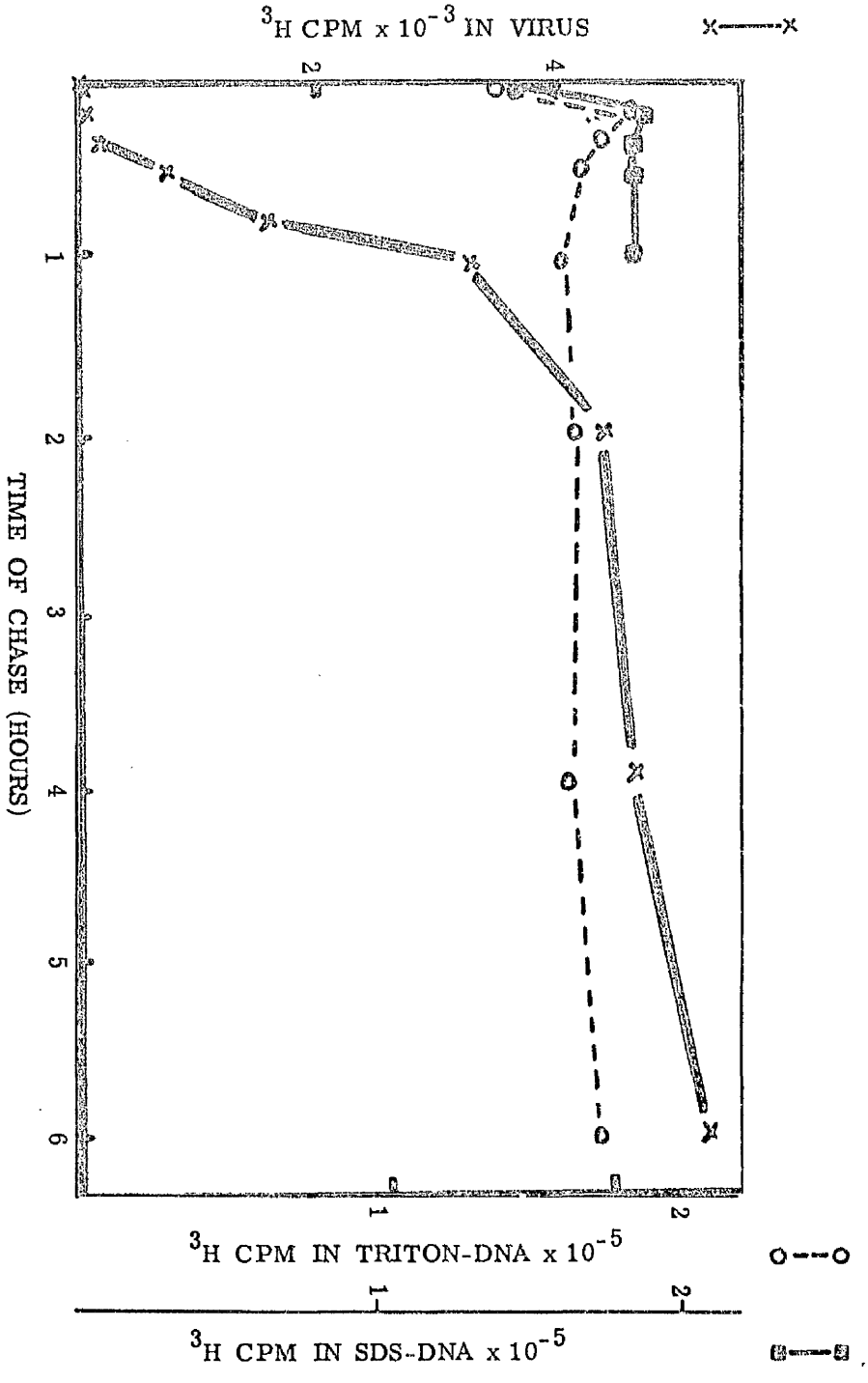

^{3}H-THYMIDINE CPM IN COMPLEX x 10^{-5}
4
8
10
20
30
40
50
60
70
80
90
TIME (HR)
1
2
^{3}H-THYMIDINE CPM IN VIRUS x 10^{-4}
5
6
7
8
LOG_{10} PFU

The complex in relation to the virus assembly process.

'Empty' shells of SV40 virus can form independently of the virus in vivo and serve as a precursor of intact virus (Ozer, 1972; Ozer & Tegtmeyer,1972). The 'empty' shells do not contain viral DNA and have a reduced content of VP4-6 compared to 'full' virus (Black, Crawford & Crawford, 1964; Estes et al,1971; Ozer, 1972; see also Table 1). It is possible that these deficiencies would be corrected if complexes of the kind reported here were to combine with 'empty' shells to yield mature virus. To further investigate this possibility that the 44S complex might be an intermediate in the viral particle assembly process, pulse-chase experiments were performed to determine whether the labelling properties of the complex are consistent with such a role.

23 monolayer cultures of BSC-1 cells were infected with 10 pfu/cell of SV40 virus and after 42 hr the medium from each culture was removed and replaced with 2ml of Eagle's medium containing 20 µCi/ml of ^{3}H-thymidine. After 10 min of incubation at 37 C, the radioactive medium was removed. The cells were washed with PBS and 20 of the dishes were overlaid with 10ml of Eagle's medium containing excess unlabelled thymidine and incubated further at 37 C. The remaining 3 cultures were either extracted with triton X-100 or with 0.5% SDS or harvested for

Fig.23. Synthesis of the complex in relation to SV40 virus assembly. 23 monolayer cultures of BSC-1 cells were infected with 10 pfu/cell of SV40 virus and after 42 hr, the medium was removed and replaced with 2 ml of warm Eagle's medium containing 20 μCi/ml of ^{3}H-thymidine. After 10 min of incubation at 37 C, the radioactive medium was removed. The cells were washed with PBS and 20 of the dishes were overlaid with 10 ml of Eagle's medium containing excess unlabelled thymidine (250 μg/ml) and incubated further at 37 C. The remaining 3 cultures were extracted with either triton X-100 or with 0.5% SDS or harvested for determination of radioactivity in virus particles (as described in the legend to Fig.28). SDS extracts were made from 4 of the cultures to which excess unlabelled thymidine had been added at 10, 20, 30, and 60 min after removal of the radioactive thymidine. Triton extracts were made from 8 of the cultures at 10, 20, 30, 60, 120, 240 and 360 min after removal of isotope. The remaining 8 cultures were harvested for determination of radioactivity in SV40 virus at 10 min, 20 min, 30 min 40 min, 60 min, 120 min, 240 min and 360 min after removal of isotope. The SDS extracts were analysed by CsCl velocity sedimentation as described in Fig.1B whereas the triton extracts were analysed by sucrose gradient centrifugation as described in Fig.22 . The quantity of radioactivity per culture sedimenting either as 21S SV40 DNA (in the SDS extracts) or as 44S SV40 44S complex or the radioactivity per culture in the form of SV40 virions was determined in each case.


^{3}H CPM x 10^{-3} IN VIRUS
TIME OF CHASE (HOURS)
^{3}H CPM IN TRITON-DNA x 10^{-5}
^{3}H CPM IN SDS-DNA x 10^{-5}

determination of radioactivity in virus particles. SDS extracts were made from 4 of the cultures to which excess unlabelled thymidine had been added at 10 min, 20 min, 30 min and 60 min after removal of the radioactive thymidine. Triton extracts were made from 8 of the cultures at 10 min, 20 min, 30 min, 60 min, 240 min. and 360 min after removal of isotope. The remaining 8 cultures were harvested for determination of radioactivity in SV40 virus at 10 min, 20 min, 30 min, 40 min, 60 min, 120 min, 360 min, and 480 min. after removal of isotope.

The SDS extracts were analysed by CsCl velocity sedimentation and the radioactivity sedimenting as 21S SV40 DNA per culture was quantitated, whereas the triton extracts were analysed by centrifugation through linear 5-20% sucrose gradients and the amount of radioactivity sedimenting as 44S complex per culture was determined in each case. The amount of radioactivity in SV40 virus at each time was also calculated. The results obtained are shown in Fig. 23.

The amount of radioactivity in the complex was observed to reach a maximum value 10 min after removal of isotope. Over the next 40 min of incubation the value was observed to decay by a factor of approximately 8%. Thereafter, the value decayed only slightly. Over the first 10 min of the chase period, the amount of DNA extractable by the SDS method was approximately equivalent to that

extractable by the triton method. However, over the next 40 min of the chase period, no decay in the quantity of radioactivity behaving as 21S SV40 DNA was observed. This indicates that the decay in triton extractable DNA observed over the period 10 min to 60 min of chase is the consequence of a portion of the DNA being converted to a form which is no longer extractable by the non-ionic detergent rather than of being degraded. Triton has no effect on SV40 virus. This portion of the DNA remains extractable by SDS, however. No radioactivity could be detected in the form of mature SV40 virus until after 10 min of the chase period. Between 20 min and 60 min of the chase period, a rapid appearance of radioactivity into virus occurs and after 1 hr of chase, the rate of increase of radioactivity in the virus over the next 5 hours. Over the 6 hour chase period, only 2% of the total SV40 DNA (as extracted with SDS) labelled during the pulse period became encapsulated into mature virus. The decay in the quantity of labelled complex was of the order of 8% over the same period. However, the bulk of the labelled complex (90%) was stable over the duration of the experiment.

The extremely low efficiency of encapsidation of the viral DNA makes it difficult to obtain conclusive evidence of a pulse-chase nature that the nucleoprotein complex is a precursor of the virus since decay of the complex is only slight. However, the

data obtained in the above experiment is consistent with the possibility that the complex could be a precursor of the full virus. The 10 to 20 min. delay in appearance of radioactivity after removal of isotope would seem to indicate a rate-limiting step in viral DNA encapsidation. The discrepancy between decay in radioactive complex (8%) and appearance of radioactivity in mature virus (2%) possibly results from low efficiency of recovery of virus from the cells.

Effect of puromycin on protein-DNA complexes

BSC-1 cells infected with SV40 virus, were exposed to tritiated thymidine in the presence of puromycin at different times after addition of the drug and protein-DNA complexes were extracted with triton X-100. A sharp reduction in incorporation of tritiated thymidine into triton-extractable SV40 DNA, similar to that observed in polyoma virus-infected cells (Bourgaux, 1972) was observed. (Table 3). Radioactive SV40 DNA was present in protein-DNA complexes which had a greatly reduced average sedimentation coefficient (25S) (Fig. 24b) compared with that of complexes obtained in the absence of puromycin (44S) (Fig. 24a). The low molecular weight DNA in Fig. 24b was observed in uninfected cells treated with puromycin and is therefore probably cellular in origin. More prolonged puromycin treatment reduced the rate of incorporation of tritiated thymidine into such

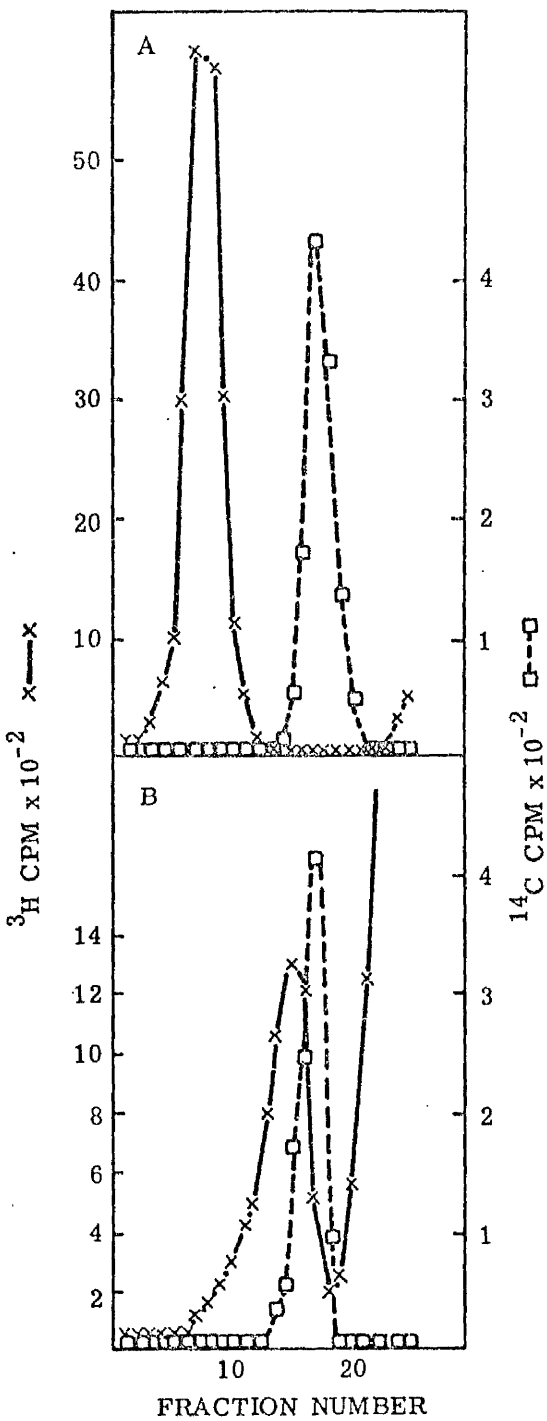
TABLE 3

Effect of puromycin on ^{3}H-thymidine incorporation into 21S SV40 DNA

Time of puromycin treatment (min)	^{3}H-c.p.m./aliquot in 21S SV40 DNA
15	10200
30	8800
60	2700
90	1500

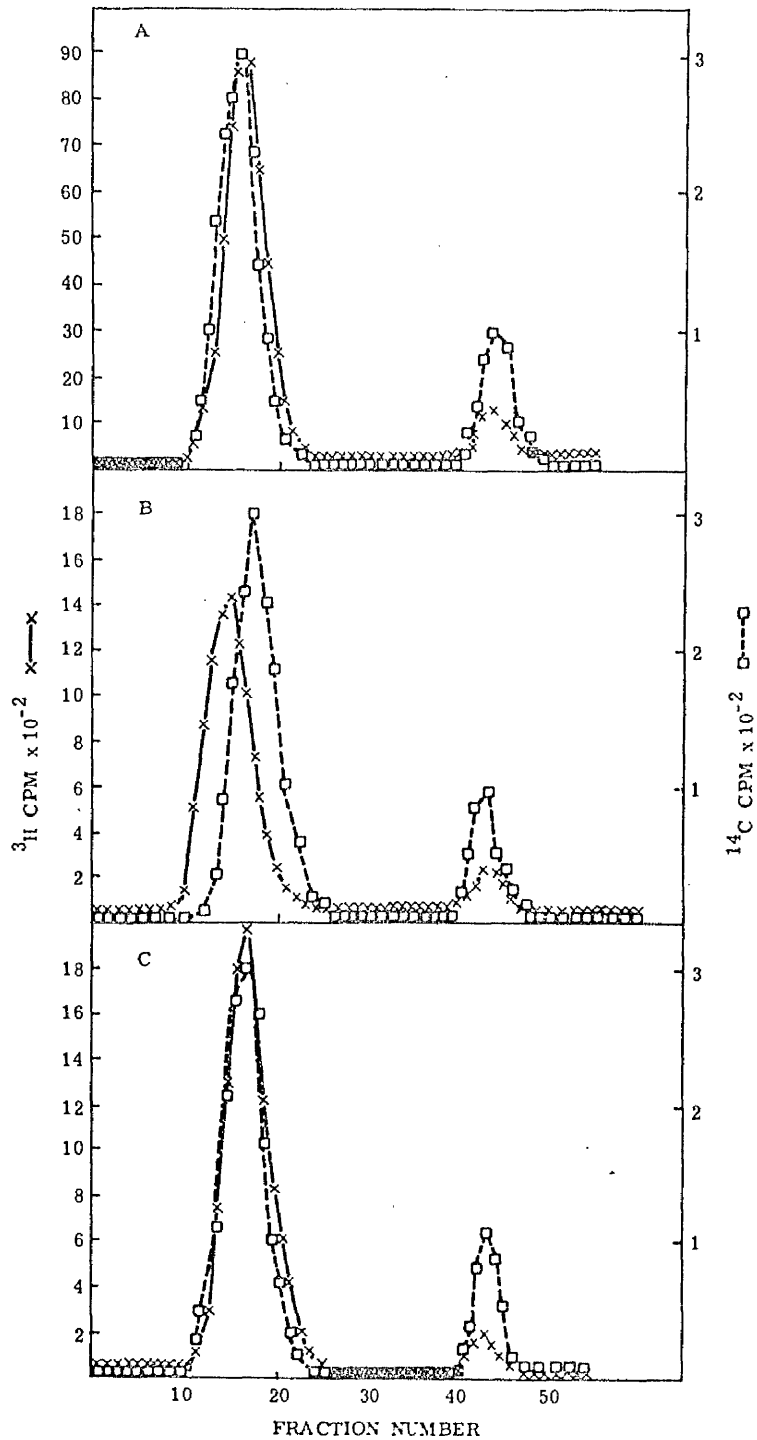
At 28 hr post-infection, 4 monolayer cultures of SV40 infected BSC-1 cells (10 pfu/cell) were treated with puromycin (final concentration 4×10^{-4}M). At 15, 30, 60 and 90 min after addition of the drug, ^{3}H-thymidine (50 μCi/ml) was added for a 30 min period. At the end of each pulse period, the cultures were extracted with triton X-100 and 0.1 ml aliquots of each extract were centrifuged for 3 hr through 3ml of CsCl (density 1.50 g/ml) in the SW50.1 rotor of a Beckman ultracentrifuge and the amount of radioactivity sedimenting as 21S SV40 DNA was determined in each case.

Fig. 24. Sedimentation analysis of protein-DNA complexes extracted from puromycin-treated (B) and untreated (A) SV40 infected BSC-1 cells. Monolayer cultures of BSC-1 cells were infected with SV40 virus (10 pfu/cell). At 28 hr post-infection, the medium was removed from the cells and replaced either with warm Eagle's medium containing puromycin (4×10^{-4}M) or with Eagle's medium containing no puromycin. After 30 min incubation at 37 C ^{3}H-thymidine was added to a final concentration of 50 μCi/ml and the cells were extracted with triton X-100 subsequent to a 30 min exposure to the isotope. Sucrose gradient sedimentation analysis of the triton extracts was performed by layering 0.2 ml samples on to 4.0 ml linear 5-20% sucrose gradients. ^{14}C-labelled 21S SV40 DNA was added as a marker. The samples were centrifuged for 50 min at 55,000 rev/min in the Spinco SW56Ti rotor at 4 C.


A
B
50
40
30
20
10
14
12
10
8
6
4
2
4
3
2
1
10
20
FRACTION NUMBER
^{3}H CPM x 10^{-2}
^{14}C CPM x 10^{-2}

complexes, but did not alter the average sedimentation coefficient. SV40 DNA which was completely free of protein was never observed after puromycin treatment. These complexes are more sensitive to treatment with deoxycholate than triton X-100. The report that protein binding to polyoma DNA in the presence of puromycin is completely inhibited (Bourgaux, 1972) may be attributable to this difference in extraction procedure. Analysis of SV40 DNA in the "puromycin complex" by dye-buoyant density gradient centrifugation indicated an increase in buoyant density and therefore a reduction in the number of superhelical turns (Fig. 25a, 25b), compared with SV40 DNA from the control extract. This change is similar to the behaviour of polyoma DNA under these conditions (Bourgaux, 1972). It is possible to interpret these results on the formation of such protein-DNA complexes in terms of specific binding events. The "puromycin DNA" in the infected cell has very few superhelical turns and has therefore a more relaxed conformation than 'normal' SV40 DNA (Upholt, Gray & Vinograd, 1971). If protein binding in these circumstances represented a random attachment of proteins present in the vicinity of the SV40 DNA, such relaxed molecules should bind a greater amount of protein and assume a higher sedimentation coefficient than 'normal' SV40 DNA (Barclay & Eason, 1972). The results presented above indicate that no such random binding processes occur.

Fig.25. Dye-buoyant equilibrium density gradient analysis of triton extracts. Triton extracts were prepared from SV40 virus-infected cells which had been exposed to ^{3}H-thymidine in the absence (A) or in the presence (B) of puromycin as described in Fig.24. A third monolayer culture of SV40 virus infected BSC-1 cells (C) was treated with puromycin and exposed to ^{3}H-thymidine as described in Fig.24. This culture was then washed free both of isotope and the drug and incubated further for 2 hr at 37 C with Eagle's medium containing thymidine (250 µg/ml). The cells were subsequently extracted with triton X-100 for analysis. Equilibrium centrifugation was performed by adding 0.4 ml samples of the appropriate triton extract to a final volume of 4.2 ml CsCl (density 1.56 g/ml)-ethidium bromide (330 µg/ml). An aliquot of ^{14}C-labelled SV40 DNA obtained by extraction of SV40 virus infected cells, labelled with ^{14}C-thymidine (1.0 µCi/ml) 24-45 hr post-infection with SDS, was added to each tube to act as a supercoiled marker. Centrifugation was carried out in a Spinco No.50 Al rotor for 40 hr at 38,000 rev/min at 20 C.


A
B
C
^{3}H CPM x 10^{-2}
^{14}C CPM x 10^{-2}
FRACTION NUMBER

Recovery from puromycin treatment.

SV40 virus infected cells were labelled with tritiated thymidine in the presence of puromycin, washed with Eagle's medium containing excess thymidine and incubated a further 2 hr. Dye-buoyant analysis of triton-extracted material demonstrated that the tritiated SV40 DNA was superimposed on the ^{14}C-labelled SV40 DNA marker (Fig. 25c). Velocity sedimentation analysis on a neutral sucrose gradient (Fig.26) revealed the presence of a protein-DNA complex with an average sedimentation coefficient of 44S. The "puromycin complex" had therefore acquired the "normal" sedimentation characteristics and its constituent DNA had the "normal" superhelix density.

To determine if DNA replication was concerned with the introduction of superhelical turns in "puromycin DNA", infected cells were labelled with tritiated thymidine in the presence of puromycin, as before. The cells were washed free of puromycin and tritiated thymidine, then incubated in the presence of 5-bromodeoxyuridine. Buoyant equilibrium analysis in CsCl indicated that about 30% of the labelled SV40 DNA had a density greater than the "light-light" ^{14}C-labelled SV40 DNA marker (Fig. 27a), which demonstrated that extensive DNA replication could occur under these conditions. When arabinofuranosylcytosine (ara-C) was added simultaneously with 5-bromodeoxyuridine no

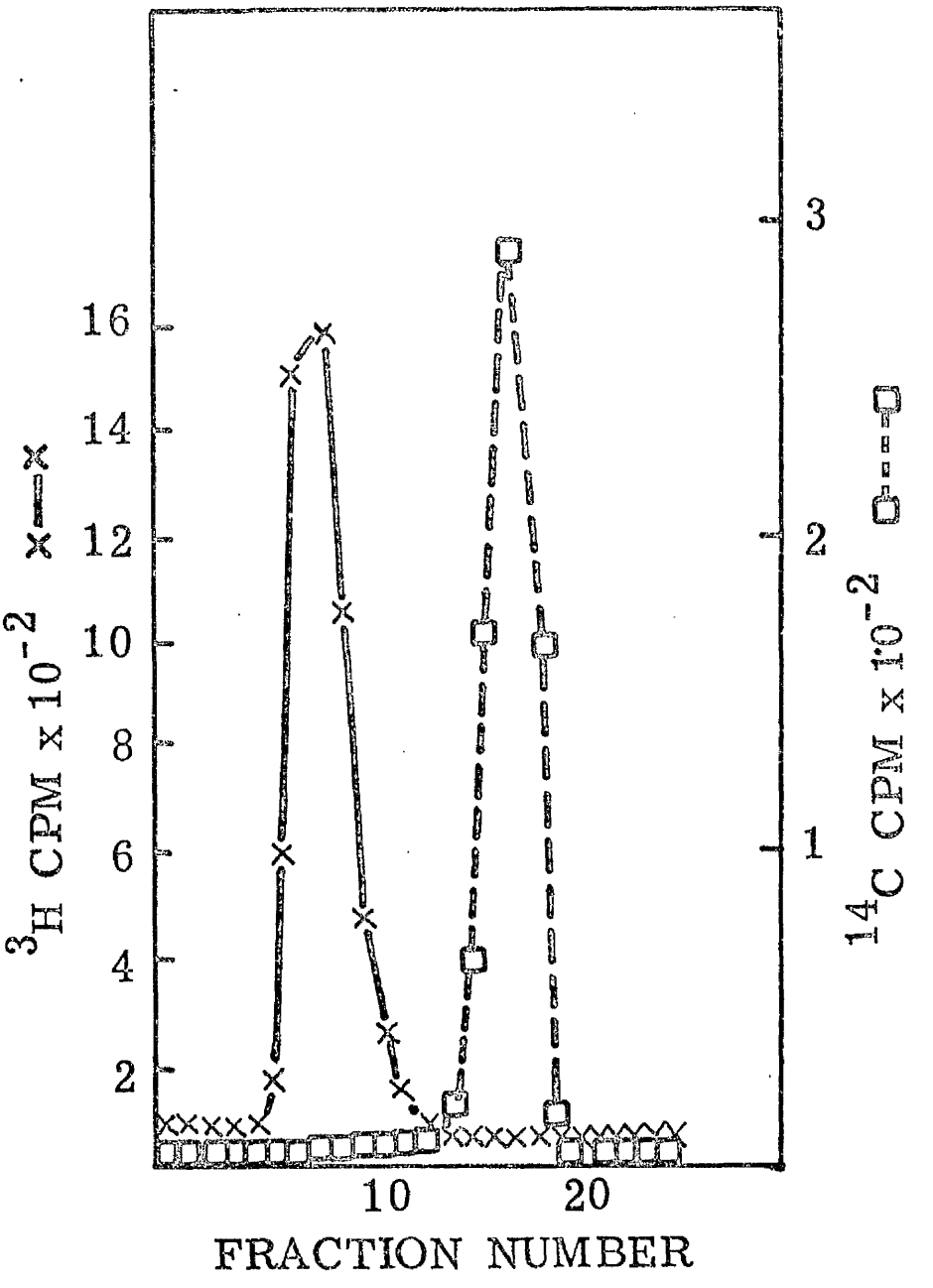
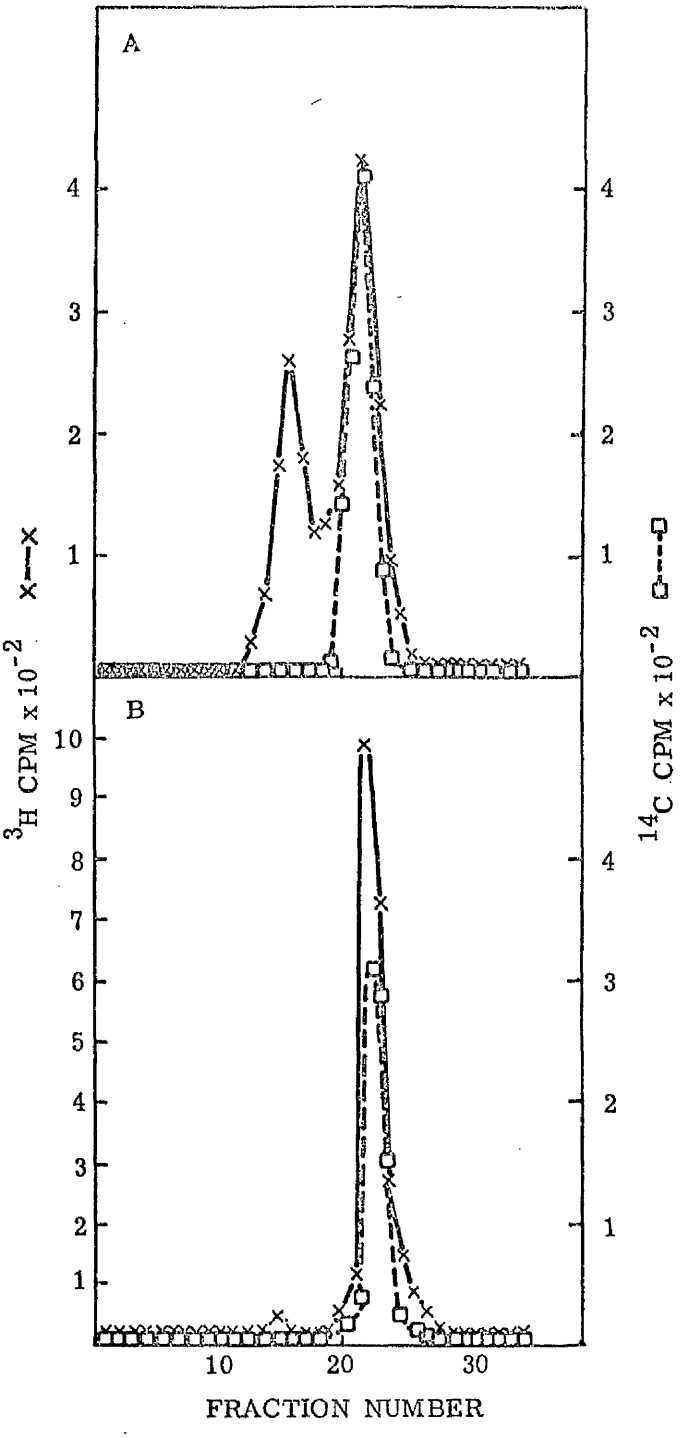


Fig. 26. Sedimentation properties of DNA-protein complexes from SV40 virus infected cells recovered from puromycin treatment. The extract analysed in Fig.25 (C) was mixed with ^{14}C-labelled 21S SV40 DNA marker and centrifuged through a 5-20% sucrose gradient as described in Fig.24.

Figure 27. Buoyant analysis of triton extracts in CsCl gradients. SV40 virus infected BSC-1 cells, which had been treated with puromycin and exposed to ^{3}H-thymidine for 30 min as described in Fig. 24, were washed free of the drug and isotope and were incubated further for 2 hr with Eagle's medium containing ara-C (28 ug/ml) (B) or no ara-C (A), 5-fluorodeoxyuridine (Calbiochem; 15 ug/ml) and 5-bromodeoxyuridine (Sigma; 5 ug/ml). The cells were extracted with triton and 0.2 ml samples were mixed with ^{14}C-labelled "light-light" SV40 DNA marker prior to equilibrium density gradient centrifugation in CsCl (density 1.75 g/ml) for 40 hr at 38,000 rev/min in the Spinco SW50.1 rotor at 20 C.

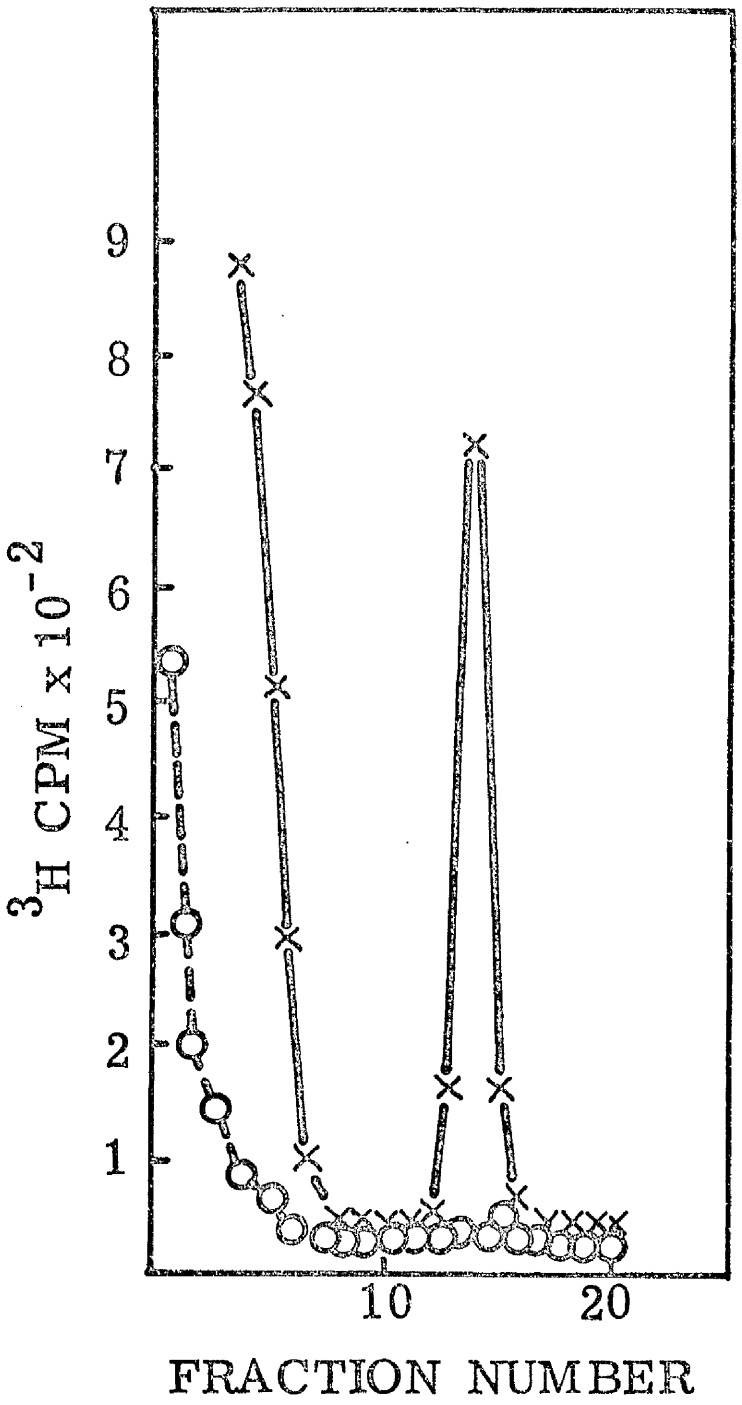

A
B
^{3}H CPM x 10^{-2} x—x
^{14}C CPM x 10^{-2} □---□
FRACTION NUMBER
10
20
30

tritium labelled DNA of hybrid density was observed (Fig.27b), indicating that DNA replication was entirely inhibited. However, when triton extracts were prepared from infected cells to which ara-C had been added, after washing free of puromycin and ^{3}H-thymidine, dye-buoyant analysis of the SV40 DNA gave results identical with those in Fig.25c. The "puromycin DNA" had therefore acquired the "normal" superhelix density. Moreover, the sedimentation properties of this extract on sucrose gradients were indistinguishable from those presented in Fig.26. The introduction of superhelical turns into "puromycin DNA" therefore appeared to occur by a nicking-reclosing process (Champoux & Dulbecco, 1972) which was independent of DNA replication. In association with this process, binding of proteins takes place. Since binding of proteins in the presence of puromycin occurs only to a limited extent, either the synthesis of these proteins has been inhibited or the production of some factor concerned with the assembly of such complexes has been inhibited. The assembly process was not, however, sensitive to ara-C.

DNA-protein complexes in virus assembly.

SV40 virus infected cells were labelled for 3 hr with tritiated thymidine in the presence of puromycin and intact SV40 virus was isolated. No radioactivity was subsequently observed in virus particles (Fig.28) despite the presence of labelled "puromycin DNA" in the cells. The amount of radioactive

Fig. 28. The effect of puromycin on SV40 virus assembly. 0.2 ml of ^{3}H-thymidine (500 µCi/ml) was added to monolayer cultures of SV40 virus-infected BSC-1 cells which had been overlayed 30 min previously (at 28 hr post-infection) with 4.0 ml of warm Eagle's medium containing either puromycin (- - o - -) (4×10^{-4} M) or no puromycin (—— x ——). After 3 hr exposure to the isotope, the cells were washed twice wirh cold saline and scraped into 2 ml of PBS. The samples were then frozen. Excess unlabelled SV40 virus infected cells were added as carrier and the mixture was adjusted to 1% with respect to sodium deoxycholate. Deoxyribonuclease and ribonuclease A were added to a final concentration of 40 µg/ml and 12 µg/ml respectively and the mixture was dispersed by sonic vibration then incubated at 37 C for 30 min. Cellular debris was removed by centrifugation at 15,000 rev/min for 30 min in an MSE 18 high speed centrifuge. The virus-containing supernatant was decanted and underlayed with 2.5 ml of saturated KBr solution and centrifuged for 3 hr at 40,000 rev/min in the Spinco SW50.1 rotor at 20 C. The fractions corresponding to the lower visible band, comprising SV40 virus, were layered onto 3.5 ml CsCl (density 1.36 g/ml) and centrifuged for 18 hr in the Spinco SW56Ti rotor at 20 C. Gradients were harvested and 5 drop fractions collected of which 50 µl were assayed for radioactivity. SV40 virus is found in the region of fraction number 14.


9
8
7
6
5
4
3
2
1
^{3}H CPM x 10^{-2}
10
20
FRACTION NUMBER

SV40 DNA synthesised under conditions of puromycin treatment was 8% of that synthesised in the absence of the drug. If 'puromycin-DNA' was encapsulated with the same efficiency as 'normal' SV40 DNA, then the amount of radioactivity behaving as mature virus from the puromycin treated culture would be 8% of that observed in the culture labelled with ^{3}H-thymidine in the absence of drug. No radioactivity was observed in full virus on analysis of puromycin treated SV40 infected cultures even when the experiment was repeated, using ten times the previous amount of radioactive thymidine. Puromycin might inhibit an event intermediate between DNA synthesis and final virus assembly. To test this possibility, infected cells were labelled with tritiated thymidine prior to puromycin treatment and virus assembly during puromycin treatment was examined. An increase of about four-fold (Table 4) in the radioactivity present in intact virus was observed over a 2 hr period. That is, virus assembly occurred under conditions of puromycin treatment although "puromycin DNA" was not encapsulated. On removal of puromycin, radioactivity which was formerly in "puromycin DNA" was recovered in intact virus particles, presumably as a result of the recovery events described above (see Fig. 29).

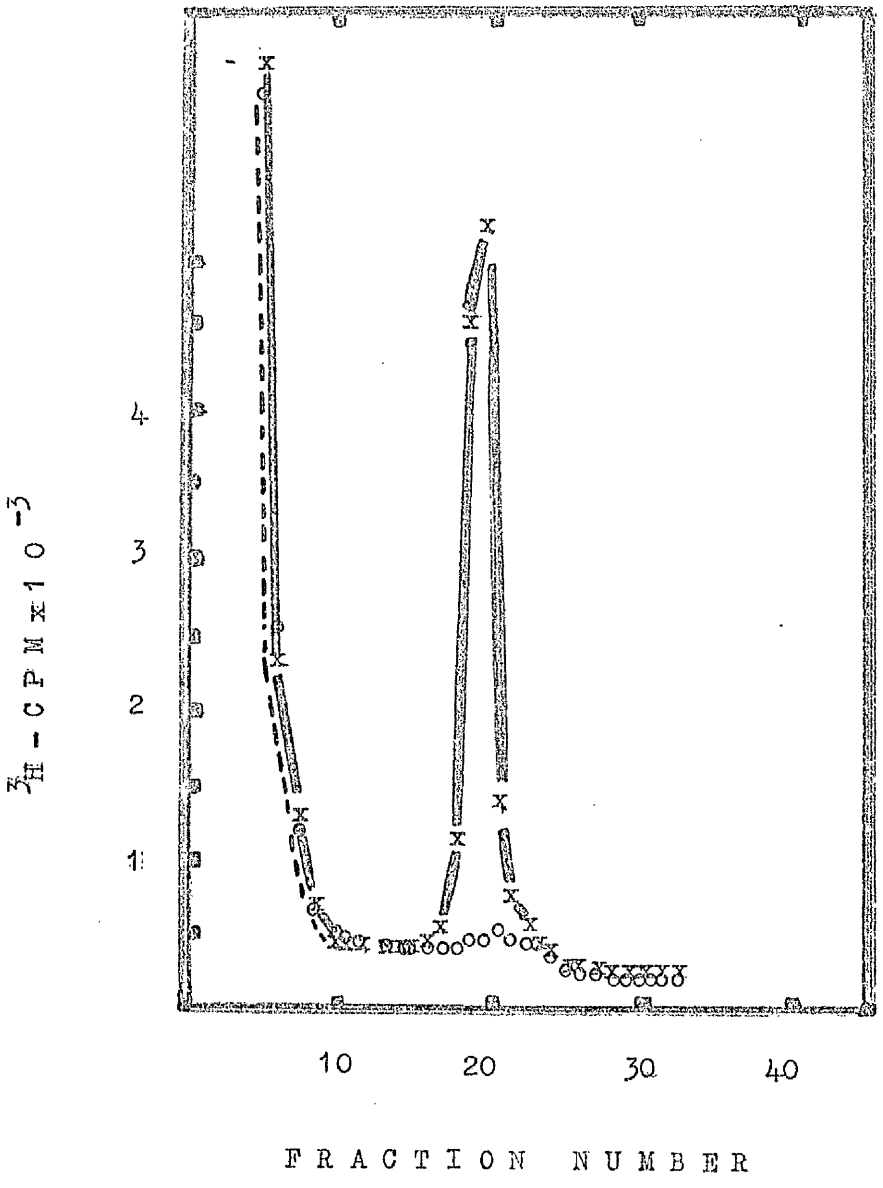
We find no evidence for exchange of proteins between preformed protein-DNA complexes and "puromycin complex" and this may be the

TABLE 4. Synthesis of SV40 virus in the presence of puromycin

Time of puromycin treatment (min)	Radioactivity in SV40 virus (cpm)
30	80
90	160
150	310

Twenty min prior to addition of puromycin, three dishes of SV40 virus infected BSC-1 cells were exposed to ^{3}H-thymidine (25 μCi/ml). After 10 min, the cells were washed free of isotope and incubated a further 10 min with Eagle's medium containing thymidine (250 μg/ml). The cells were then (28 hr post-infection) treated with puromycin (4×10^{-4} M) and plates were harvested at 30, 90 and 150 min thereafter as described in Fig.28. Determination of the radioactivity of SV40 virus ineach sample was determined as described in Fig.28.

Fig.29. DNA encapsulation after recovery from puromycin treatment. At 28 hr post-infection, 2 monolayer cultures of SV40 infected (10pfu/cell) BSC-1 cells were labelled with ^{3}H-thymidine (50 μCi/ml) for 30 min in the presence of puromycin (4×10^{-4}M). The medium was then removed from both cultures and replaced with warm Eagle's medium containing either puromycin (4×10^{-4}M) (x ---- x) or no puromycin (o - - - o) and excess unlabelled thymidine (250 μg/ml). The cultures were then further incubated for 5 hr. The cells were then harvested and the amount of radioactivity in SV40 virions was determined in each case. Fractions from the equilibrium gradient were collected directly onto filter discs.


^{3}H - CPM x 10^{-3}
4
3
2
1
10
20
30
40
FRACTION NUMBER

result of co-operative binding of certain proteins to DNA (Barclay & Eason, 1972; Rubin & Moudrianakis, 1972). "Puromycin DNA" has an altered superhelix density (Bauer & Vinograd, 1968) due, it appears to restricted binding of protein. It is probable that binding of protein to SV40 DNA is required to produce a condensed structure suitable for encapsulation and that steric restrictions prevent packaging of "puromycin complex".

DISCUSSION

DISCUSSION

The experiments reported in this work show that when SV40 infected BSC-1 cells are exposed to radioactive thymidine, all forms of labelled SV40 DNA can be extracted in the form of viral DNA-protein complexes. After labelling for long periods with ^{3}H-thymidine, a 44S complex was present which contained only 21S SV40 DNA. When shorter pulse times were used, DNA-protein complexes with higher S values were present, in addition to 44S material, and which contained replicative form SV40 DNA. In a similar study, Green et al (1971) have demonstrated that polyoma DNA-protein complexes can be extracted from mouse embryo cells infected with the virus. In both studies, free viral DNA was never observed using the triton extraction procedure.

The conclusion that the altered sedimentation properties of the forms of SV40 DNA in the infected cell are due to protein binding is based on the sensitivity of the complex to protease

digestion and on labelling studies with labelled amino acids. Interpretation of the protein labelling experiments were complicated due to the presence of radioactive ribonucleoproteins of cellular origin. However, after digestion with ribonuclease, a virus-specific component labelled with ^{14}C-amino acids was more clearly evident in a position corresponding to that of ^{3}H-labelled SV40 DNA in double label experiments. The double-label experiments do not conclusively demonstrate, however, that the labelled protein in the 44S component is bound to the viral DNA and it may be that the proteins represent some other virus-induced component which fortuitously cosediments with the ^{3}H-labelled complex. This possibility might be explored by means of alternative purification and fractionation techniques.

A procedure which permits radiochemical purification of SV40 DNA-protein complexes from infected cells, and which thereby allows identification of the constituent polypeptides, has been described. A nuclear extraction procedure gave yields of nucleoprotein complex equivalent to about 40% of that obtained from whole cells. Actinomycin D was found to remove a ribonucleoprotein complex which had similar sedimentation properties as the SV40 DNA-protein complex and which had been difficult to eliminate by other methods. It is possible that the protein content of the complex might have been affected by this drug, since puromycin has

drastic effects in this respect. However, synthesis of the complex were found to be completely unaffected by the levels of drug employed. Further studies have indicated that the sedimentation properties of the complex are entirely unaffected by actinomycin concentrations as high as 2.0 µg/ml, although the rate of synthesis of the complex begins to decrease. The protein content of the complex cannot therefore be grossly modified by actinomycin D.

The most striking feature of the analysis of the proteins in SV40 DNA-protein complexes from infected cells is the content of proteins in the region usually designated to contain VP4, 5 and 6. Indeed, the patterns are similar in many respects to those obtained from virus structural complexes prepared from purified virus (Anderer et al 1968; Huang et al 1972). We find that VP2 and VP3 are clearly defined components with additional peaks around molecular weight 80,000, 28,000 and possibly about 22,000. Problems associated with especially tight binding of VP3 to SV40 DNA reported by Huang et al (1972) were not evident in this work. These authors have suggested that VP1 may be detected in virus structural complex preparations since it is present in large amounts in alkali-degraded virus preparations and may therefore be difficult to remove entirely. A similar explanation might be

conceivably adopted here to account for the presence of VP1 in the complexes prepared from nuclei, where virus assembly occurs and where amounts of VP1 would be correspondingly high.

In an effort to demonstrate that the complexes do not appear in the course of extraction, due to non-specific binding of protein to virus DNA, reconstruction experiments were performed in which purified 21S SV40 DNA was incubated with triton extracts. The sedimentation properties of the virus DNA were unaltered by such treatment and no complexes were present. Since these experiments cannot duplicate precisely the conditions in the cell at the time of extraction some form of non-specific interaction cannot, on this basis alone, be excluded. However, SV40 DNA synthesised in the presence of puromycin has very few superhelical turns and, therefore a more relaxed conformation than 'normal' SV40 DNA. If protein binding in these circumstances represented a random attachment of proteins to SV40 DNA, such relaxed molecules should bind a greater amount of protein and assume a higher sedimentation coefficient than 'normal' SV40 DNA. Since this was not observed to be the case, such random binding processes do not occur. SV40 'empty' shells can form independently of the virus *in vivo* and can serve as a precursor of intact virus (Ozer, 1972; Ozer & Tegtmeyer, 1972). The principle of 'empty' shells acting as intermediates in virus assembly has been established in the case of both poliovirus (Jacobson & Baltimore, 1968) and for the

bacteriophage T4 (Wood et al 1971).

SV40 'empty' shells possess all of the structural proteins observed to be present in the full virus but do not contain viral DNA and are deficient in the proteins VP4, 5 & 6 (Black, Crawford & Crawford, 1964; Ozer, 1972; Estes et al, 1971). These deficiencies would be corrected if the nucleoprotein complex were to combine with 'empty' particles to yield mature virus. However, the stoichiometry is not simple since the 'empty' shells are morphologically heterogeneous (Ozer, 1972) and the nucleoprotein complex described here contains proteins not found in the mature virus.

It is difficult to obtain clear evidence that the 44S complex is a precursor of the virus since the efficiency of encapsidation of intracellular SV40 DNA is very low. The consequence of this is that decay of complex is very slight when appropriate pulse-chase experiments are performed. The data obtained, however, is compatible with the possibility that the complex, to a small extent, is a precursor of virus. It has not been possible to detect intracellular unencapsidated SV40 DNA in any form other than as a 44S DNA-protein complex and, furthermore, this form of the viral DNA accounts for all the newly-synthesised unencapsidated viral DNA in the cell.

On this basis therefore, there appears to be no evidence

for an alternative to the complex as the source of encapsidated viral DNA. Indeed, protein binding to viral DNA appears to be an essential step in the process of SV40 viral particle assembly. SV40 DNA synthesised in the presence of puromycin was found to be present in the form of a DNA-protein complex (25S) in which the binding of protein had been severely restricted and was found to have very few superhelical turns. Puromycin inhibits the appearance of ^{3}H-thymidine into mature virus particles, despite the presence of labelled 'puromycin DNA' in the cells. Experiments were performed which demonstrated that virus particle assembly occurs under conditions of puromycin treatment, but that 'puromycin DNA' was not encapsidated. If the puromycin is subsequently removed, the SV40 DNA was observed to recover both its 'normal' protein complement (44S) and its 'normal' superhelix density and can be subsequently encapsidated. Since these recovery processes can take place in the presence of ara-C, it appears that the introduction of superhelical turns into SV40 DNA can occur by a nicking-reclosing process which is independent of DNA replication and that in association with this process, binding of proteins takes place. It is probable that binding of proteins to SV40 DNA is required to produce a condensed structure for encapsidation and that steric restrictions prevent packaging of the puromycin complex. However, since polyoma virus assembly may involve a preliminary interaction with nucleoprotein

complex with capsid protein (Friedmann, 1972), the participation of the proteins in the complex is likely to be more complex than solely to induce conformational changes in the viral DNA. The small amounts of VP1 in the complex may be significant in a possible interaction of complex with 'empty' particles.

Proteins designated as possible intermediate structural elements have been VP3 in the case of SV40 virus (Huang et al, 1972) and VP6 in the case of polyoma virus (Friedmann,1972). Since it has been found that all proteins are present in both 'empty' shells and in the SV40 DNA-protein complex, it is difficult to select which, if any, of the proteins are essential assembly factors.

There is evidence that the binding of basic proteins to adenovirus DNA is an important step in the process of virus assembly. Adenovirus is in the form of an icosahedron of diameter 70 nm with 252 sub-units or capsomeres (Horne et al, 1959; Valentine et al, 1965). Two types of capsomere have been recognised and designated as hexons and pentons (Ginsberg et al, 1966). The hexons have 6 nearest neighbours and there are 240 situated on the faces and edges of the icosahedron. The pentons are composed of a base and a fibre with a knob on the end and they are situated at the 12 apices of the icosahedron. The components hexons, pentons and fibres may be obtained as soluble antigens in virus-infected cells and purified by chromatography (Klemperer et al, 1959). The

DNA is present in an internal core of diameter 25 - 35 nm (Morgan et al, 1959; Epstein et al, 1960). Treatment of the viral particle with acetone released the internal core of the virus which could be separated from capsomeres by zonal rate centrifugation in a sucrose gradient (Laver, Suriano & Green, 1967). The proteins associated with the adenovirus DNA accounted for at least 20% of the total virion protein. These proteins were relatively rich in arginine and resembled histones (Russell, Laver & Sanderson, 1968). Adenovirus failed to mature when cells were infected in the absence of arginine (Rouse & Schlesinger. 1967). Arginine deprivation resulted in the synthesis, but not the assembly of most of the viral proteins. These proteins were assembled into mature virions containing DNA after arginine was restored (Everitt, Sandquist & Philipson, 1971). The only antigen the synthesis of which appeared to be dependent on a supply of exogenous arginine was the internal or 'core' antigen (Russell & Becker, 1968).

Short term labelling experiments indicated the presence of nucleoprotein complexes which contained replicative form DNA. The properties and kinetics of conversion of this 25S DNA to 21S DNA are compatible with the results reported in AGMK cells infected with SV40 (Levine et al, 1970) and in polyoma-infected ME cells (Bourgaux et al, 1969). The 25S SV40 DNA present in rapidly-sedimenting complexes could be chased into the 21S SV40

DNA present in the 44S material.

The very high sedimentation coefficient (90S) of the complex containing replicative form DNA suggested that the 44S complex acted as a template for the synthesis of the progeny strands in the replicative intermediate. However, precise analysis of the sedimentation distributions obtained for the nucleoprotein complexes is difficult to perform. Changes in sedimentation behaviour could arise from changes in molecular weight, from changes in conformation, or from both of these simultaneously.

The origins and nature of the proteins in the 90S replicating complex have not been established, nor is it known what roles the bound proteins play in the replication process per se. It has recently been reported by Green (1972) that the polyoma nucleoprotein complex contains no endogenous DNA polymerase activity and it would therefore appear that at least some of the proteins present in the 90S replicating complex are bound temporarily to the DNA and presumably dissociate after replication is complete.

The role of the individual protein components present in the 44S DNA-protein complex has not been clearly established, but it is clear that association of protein with SV40 DNA at the time of ring closure has important consequences in terms of the superhelix

density of the closed circular daughter molecules. If different amounts of protein, or a variable number of types of protein were bound, it might then be possible to account for the superhelix density heterogeneity which exists in intracellular 21S SV40 DNA in infected cells (Eason & Vinograd, 1971).

The 3 smallest proteins (VP4-6) of SV40 or of Polyoma (VP5-7) contain no tryptophan, and have lysine and arginine contents characteristic of histones (Robin, Harle & Dulbecco, 1971; Bourgaux, personal communication data for Polyoma; B. Hirt personal communication, data for SV40). Frearson & Crawford (1972) have demonstrated that, in the case of Polyoma virus, the peptide maps of these basic proteins, after labelling with ^{35}S-methionine, are similar to those of uninfected host cell histones. Similar results have been obtained in the case of SV40 virus (B. Hirt, Cold Spring Harbour Tumour Virus Meeting August 1972). A wealth of evidence (Stellwagen & Cole, 1969) suggests that the binding of histones to DNA inactivates transcription and that these proteins act as regulatory molecules in gene expression. SV40 'core' complex, obtained on alkaline dissociation of virus particles (Anderer et al 1967, Huang et al, 1972), resembles the 44S nucleoprotein complex in that it has a similar sedimentation coefficient, similar salt and detergent sensitivity, and similar protein composition. The 'core' complex is active in DNA-dependent RNA synthesis with

both E. Coli DNA-dependant RNA polymerase and an RNA polymerase from mammalian cells (Huang, Nonoyama & Pagano, 1972). However, the template activity is much less than that of an equal amount of deproteinised SV40 component 1 DNA. DNA-RNA competition hybridisation experiments showed that the RNA synthesised in vitro with the 'core' complex as template hybridised with only a fraction of the 21S viral DNA transcribable with the E. Coli RNA polymerase. Viral protein appears to prevent transcription of some of the viral sequences. However, the function of the proteins in the 44S nucleoprotein complex in terms of viral gene expression is not at present known, but on the basis of the close resemblance of the 44S complex to the 'core' complex, research directed at the elucidation of such functions would appear to be a promising field of study.

REFERENCES

REFERENCES

Aloni,Y. (1972) Proc. Natl. Acad. Sci. U.S.A. 69 2404.

Aloni,Y., E. Winocour & L. Sachs (1968) J. Mol. Biol. 31 415.

Anderer, F.A., M.A. Koch & H.D. Schlumberger (1968) Virology 34 452.

Anderson, C. & R. Gesteland (1972) J. Virology 9 758.

Barban, S. & R. Goor (1971) J. Virology 7 198.

Barbanti-Brodano G., Swetly,P. & H. Koprowski (1970) J. Virology 6 78

Barclay, A. & R. Eason (1972) Biochim. Biophys. Acta 269 37

Bauer, W. & J. Vinograd (1968) J. Mol. Biol. 33 141.

Black, P.H., W.P. Rowe, H.C. Turner & R.J. Huebner (1963)
Proc. Natl. Acad. Sci. U.S.A. 50 148.

Black, P.H. (1968) Ann. Rev. Microbiol 22 391.

Black, P.H., Crawford, L.V. & Crawford E.M. (1964) Virology 24 388.

Blackstein, M.E., C.P. Stanners & A.J. Formilo (1969)
J. Mol. Biol. 42 301.

Bourgaux, P., D. Bourgaux-Ramoisy & R. Dulbecco (1969) Proc.
Natl. Acad. Sci. U.S.A. 64 701.

Bourgaux, P. & D. Bourgaux-Ramoisy (1971) J. Mol. Biol. 62 513.

Bourgaux, P. & D. Bourgaux-Ramoisy (1972) Nature New Biol. 235 105.

Bonner, J.T., Chalkley, G.R., & Dhamus, M., "Methods in Enzymology"
(S.P. Colwick & N.O. Kaplan eds.) Vol.12 part B p23-24.
Academic Press, New York.

Butler, J.A.V., Johns, E.W. & Phillips, D.M.P. (1968)
Progr. Biophys. Mol. Biol. 18 211.

Cairns, J. (1963) Cold Spring Harbor Symp. Quant. Biol 28 43 .

Cairns, J. (1966) Scientific American 216 37.

Caspar D.L.D. & A. Klug (1962) Cold Spring Harbor Symp. Quant.
Biol. 27 1.

Carp, R.I., Sauer, G., & F. Sokol (1969) Virology 37 214.

Champoux, J.J. & R. Dulbecco (1971) Proc. Natl. Acad. Sci.U.S.A.
69 143.

Crawford, L.V. & E.M. Crawford (1963) Virology 21 258.

Crawford, L.V., Crawford, E.M. & Watson D.H. (1962) Virology 18 170.

Crawford, L.V. & P.H. Black (1964) Virology 24 388.

Crawford, L.V. & M.J. Waring (1967) J. Mol. Biol. 25 23.

Dulbecco, R. & M. Vogt (1963) Proc. Natl. Acad. Sci. U.S.A. 50 236.

Dulbecco, R., L.W. Hartwell & M. Vogt (1965) Proc. Natl. Acad.
Sci. U.S.A. 53 403.

Danna, K.J. & Nathans D (1972) Nature New Biology 236 200

Danna, K.J. & Nathans, D. (1973) Proc. Natl. Acad. Sci. U.S.A.

Eagle, H. (1959) Science 130 432

Eason, R. & Vinograd, J. (1971) J. Virology 7 1.

Eckhart, W. (1968) Physiol. Rev. 48 513.

Eddy, B.E., G.S. Borman, G.E. Grubbs & R.D. Young (1962)
Virology 17 65.

Epstein, M.A., Holt, S.J. & Powell, A.K. Brit. J. Exp. Path. 4 567.

Estes, M., Huang, E-S, & J. Pagano (1971) J. Virology 7 636.

Everitt, E.B., B. Sandquist & L. Philipson (1971) J. Virology 8 742.

Fareed, G.F., & N.P. Saltzman (1972) Nature New Biol 238 275.

Fareed, G.F. & N.P. Saltzman (1972a) J. Virology 10 484.

Follett, E.A.C. & L.V. Crawford (1968) J. Mol. Biol. 34 565.

Frearson, P.M. & L.V. Crawford (1972) J. Gen. Virol. 14 141.

Friedmann, T. & D.David (1972) J. Virology 10 776.

Gilbert, W. & D. Dressler (1968) Cold Spring Harbor Symp. Quant.
Biol. 33 473.

Ginsberg, H.S., H.G. Periera, R.C. Valentine & W.C. Wilcox (1966)
Virology 28 782.

Gershan, D. L. Sachs & E. Winocour (1966) Proc. Natl. Acad. Sci.
U.S.A. 56 918.

Green, M (1970) Ann. Rev. Biochem. 39 735-756.

Green, M.H., H. Miller & S. Hendler (1971) Proc. Natl. Acad. Sci.
U.S.A. 68 1032

Green, M.H. (1972) J. Virology 10 32.

Hirai, K. & V. Defendi (1972) J. Virology 9 705

Habel, H. & B.E. Eddy (1963) Proc. Soc. Exp. Biol. Med. 113 1

Hirt, B. (1966) Proc. Natl. Acad. Sci. U.S.A. 55 997.

Hirt, B. (1967) J. Mol. Biol. 26 365

Hirt, B. (1969) J. Mol. Biol. 40 141.

Hirt, B. (1972) PERSONAL COMMUNICATION ~~Cold Spring Harbor Tumour Virus Meeting, Aug~~

Hsiung, G.D. & Gaylord, W.H. Jr. (1961) J. Exptl Med. 114 975

Horne, R.W. S. Brenner, A.P. Waterson & P. Wildy (1959) J. Mol. Biol. 1 84.

Huang, E.S., M.K. Estes and J.S. Pagano (1972) J. Virology 9 923.

Huang, E.S. M. Nonoyama and J.S. Pagano (1972) J. Virology 9 930.

Hudson, B., W.B. Upholt, J. Devinney & J. Vinograd (1969) Proc. Natl. Acad. Sci. U.S.A. 62 813.

Jacobson, M.F, & D. Baltimore (1968) J. Mol. Biol. 33 369.

Jaenisch, R. A. Mayer & A.J. Levine (1971) Nature New Biol. 233 72.

Kit, S. D.D. Dubbs, P. Frearson & J. Melnick (1966) Virology 29 69.

Kit. S., L.J. Piekarski & D.R. Dubbs (1967) J. Gen Virol. 1 163.

Kit, S., R.A. Detorres, D.R. Dubbs & M.L. Salvi (1967) J. Virology 1 738.

Kit, S., Kurimura, K., Detorres, R., & D.R. Dubbs (1969) J. Virology 3 25.

Klemperer, H.G., & Periera H.G. (1959) Virology 9 536.

Klug, A. J. Mol. Biol. (1965) J. Mol. Biol. 11 424.

Knippers, J.M Whalley & Sinsheimer, R. (1969) Proc. Natl. Acad. Sci. U.S.A. 64 275.

Koch, M.A. H.J. Eggers, F.A. Anderer (1967) Virology 32 503.

Lavi, S. & Winocour, E., (1972) J. Virology 9 309.

Levine, A.J., H.S. Kang & F.E. Billheimer (1970) J. Mol. Biol. 50 549.

Levine, A.J., H.S. Kang, T.B. Fsbach & D.A. White (1971) J. Virology 7 112.

Levine, A.J. & A.K. Teresky (1970) J. Virology 5 451.

Lewis, A.M. & W.P. Rowe (1971) J. Virology 7 189.

Martin, M.A. & D Axelrod (1969) Proc. Natl. Acad. Sci. U.S.A. 64 1203

Martin, M.A. (1970) Cold Spring Harbor Symp. Quant.Biol. 35 833.

Mattern, C.F.T., Takemoto, K.K., & Deleva, A.M., Virology 32 511 (1967.

Melnick, J.L. (1962) Science 135 1128.

Michel, M., Hirt, B., & Weil, R. (1967) Proc. Natl. Acad. Sci. U.S.A. 58 1381.

Morgan,C. & Rose, H.M. (1959) 9th Symp. Soc. Gen. Microbiol. 256

Oda, K. & Dulbecco, R. Proc. Natl. Acad. Sci. U.S.A. (1968) 60 525.

Okazaki,R., Okazaki,T., Sakabek K, Sugimoto, K. & Sugino, K. (1968) Proc. Natl. Acad. Sci. U.S.A. 59 598.

Okazaki,R., Okazaki, T., Sakabek, K. Cold Spring Harbor Symp. Quant. Biol. (1968a) 33 129.

Ozer, H.L. (1972) J. Virology 9 41.

Ozer, H.L. & P. Tegtmeyer (1972) J. Virology 9 52.

Ozanne, B., P.A. Sharp & J.F. Sambrook (1973) J. Virology In Press.

Radloff, R.W., W. Bauer & J. Vinograd (1967) Proc. Natl. Acad. Sci. U.S.A. 57 1514.

Ritzi, S. & Levine, A.J. J. Virology 5 686.

Roblin, R., E. Harle & R. Dulbecco (1971) Virology 45 555.

Robson, A.S. O'Connor, G.T., Kirchstein R.L. & Branigan, W.J. (1962) J. Natl. Cancer Inst. 29 765.

Rouse, H.C., & R.W. Schlesinger (1967) Virology 33 513 .

Russell, W.C., W.G. Laver & P.J. Sanderson (1968) Nature 219 1127.

Russell, W.C. &Y. Becker, (1968) Virology 35 18.

Rubin, R.L. & E.N. Moudrianakis (1972) J. Mol. Biol. 67 361.

Sambrook, J.F., Westphal, H., Srinivasan, P.R. & Dulbecco, R. (1968) Proc. Natl. Acad. Sci. U.S.A. 60 1288.

Sambrook, J.F., Sharp, P.A. & Keller, W. (1972) J. Mol. Biol. 70 57.

Sauer, G. &Kidwai M, (1968) Proc. Natl. Acad. Sci. U.S.A. 61 1256.

Sauer, G. (1971) Nature New Biology 231 135.

Sauer, G. & Fischer, H. (1972) J. Virology 9 1

Sebring, E.D., T.J. Kelly, M.M. Thoren & N.P. Salzman. (1971) J. Virology 8 478.

Shapiro, A.L., Vinuela, E. & Maizel, J.V. Biochem. Biophys. Res. Comm. 28 815.

Stellwagen, R.H. & R.D. Cole (1969) Ann. Rev. Biochem 38 712

Sweet, B.H. & Hillman, M.R. (1960) 2nd International Conference on Live Poliovirus Vaccines, Washington D.C. Panam Health Org. & W.H.O. 79.

Sweet, B.H. & Hillman, M.R. (1960b) Proc. Soc. Exptl. Biol. Med. 105 420.

Tai, H.T., C.A. Smith, P.A. Sharp & J. Vinograd (1972) J. Virology 9 317

Tevethia, S.S., M. Katz & F. Rapp (1965) Proc. Soc. Exptl. Biol. Med. 119 896.

Tevethia, S.S., G.T. Diamandopoulos, F. Rapp & J.F. Enders (1968) J. Immunol. 101 1192.

Thoren, M.M., E.D. Sebring & N.P. Saltzman (1972) J. Virology 10 462

Thorne, H.V., Evans, J., & Warden, D, (1968) Nature 219 728

Tomizawa, R., & K. Ogawa (1968) Cold Spring Harbor Symp. Quant. Biol. 33 533.

Tonigawa, S., Walter,G., Bernardini, A., & Dulbecco, R. (1970) Cold Spring Harbor Symp. Quant. Biol. 35 823.

Uchida, S., Yoshiike, K. & Watanabe, S. (1968) Virology 34 1

Uchida, S., & Watanabe (1968) Virology 35 166.

Upholt, W.B., Gray, H.B. Jr. & Vinograd, J. J. Mol. Biol. (1971) 62 21.

Valentine, R.C., & Pereira, H.G. (1965) J. Mol. Biol. 13 13.

Vinograd, J., J. Lebowitz, R.W. Radloff, R. Watson, & P. Laipis. (1965) Proc. Natl. Acad. Sci. U.S.A. 53 1104

Wang, J.C., Baumgarten, D. & Olivera, B.M. (1967) Proc. Natl. Acad. Sci. U.S.A. 58 1852

Weil, R., Michel, M., & Ruschman, G., (1965) Proc. Natl. Acad. Sci. U.S.A. 53 1468.

Weil, R. & Vinograd, J. (1963) Proc. Natl. Acad. Sci. U.S.A. 50 730.

Westphal, H. & R. Dulbecco. (1968) Proc. Natl. Acad. Sci. U.S.A. 59 1188.

Westphal, H. (1970) J. Mol. Biol. 50 407.

Winocour, E., A.M. Kaye & V. Stollar (1965) Virology 27 156.

Wood, W.B., Luftig, R.B. & Okinaka, R. (1971) J. Mol. Biol. 57 555

Yoshiike, K. (1968) Virology 34 391.

SUMMARY

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SV40 deoxyribonucleic acid (DNA) can be extracted with triton X-100 from productively infected cells in the form of a DNA-protein complex which is quite distinct from the virus and which has a sedimentation coefficient of 44S.

Analysis of the proteins present in the complex is complicated by the presence in the extracts of large amounts of a ribonucleoprotein component with a sedimentation coefficient close to that of the complex. However, a nuclear extraction procedure coupled with the use of actinomycin D abolished the synthesis of the ribonucleoprotein component and, as a consequence of this, radiochemical purification of ^{3}H-leucine labelled complex could be achieved. Analysis of ^{3}H-leucine labelled complex on sodium dodecyl sulphate-polyacrylamide gels revealed that the complexcontained all of the Virus Capsid proteins (1,2,3,4,5, and 6) but in greatly altered relative proportions from those observed in mature virus particles. The complex appears to be particularly rich in the small basic proteins (4,5 and 6). SV40 'empty' particles possess all of the structural proteins observed to be present in SV40 virus but do not contain viral DNA and are deficient in proteins 4,5, and6. The above deficiencies would be corrected

if the complex were to combine with an 'empty' particle to yield mature virus. Pulse-chase experiments with ^{3}H-thymidine provided further data which was consistent with the possibility that the complex is a precursor of mature virus.

Short term labelling experiments with ^{3}H-thymidine demonstrated that SV40 DNA molecules in the course of replication were also present as DNA-protein complexes (90S).

SV40 DNA synthesised in the presence of puromycin was found to be present in a DNA-protein complex which had a greatly reduced sedimentation coefficient (25S) and also to have very few superhelical turns. If the puromycin is subsequently removed, the SV40 DNA is observed to recover both its normal protein complement (44S) and its normal superhelix density. Since this recovery process can take place in the presence of arabinocytosinylfuranoside (a potent inhibitor of SV40 DNA replication), it therefore appears that introduction of superhelical turns into SV40 DNA occurs by a nicking-reclosing process which is independent of DNA replication and that in association with this process, binding of proteins takes place.

Puromycin inhibits the appearance of ^{3}H-thymidine into mature virus particles, despite the presence of labelled 'puromycin-DNA' in the cells. Experiments were performed which demonstrated that virus particle assembly occurs under conditions of puromycin treatment, but that 'puromycin-DNA' was not encapsidated.

It is probable that binding of protein to SV40 DNA is required to produce a condensed structure for encapsidation and that steric restrictions prevent packaging of the 'puromycin complex'.

SUMMARY OF THE THESIS TO BE PRESENTED FOR THE DEGREE OF PH.D.

BY

MARTIN WHITE

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