A Study of Nucleic Acid Synthesis in Mammalian Cells infected with Herpes Simplex Virus.

by

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John Hay, B.Sc.

The aim of this thesis was to investigate the metabolism of DNA and RNA in mammalian cells before and after infection with Herpes Simplex virus.

In furtherance of this aim, a method was developed for the extraction of DNA from Herpes Simplex virus, and from its host cell, BHK 21 (C13). This method was also applied successfully to a variety of DNA-viruses and mammalian cells.

The isolated viral DNA was demonstrated to be biologicall active in certain transformation experiments, to be relatively homogeneous with respect to size, and to have the characteristic melting profile of a double-stranded DNA molecule. Suitable conditions for the irreversible separation of the component strands of viral DNA were established.

Bouyant density measurements in CsCl and melting temperat determinations on viral and host DNAs indicated that the base compositions of these DNAs are, respectively, 68-69% guanine plus cytosine and 41% guanine plus cytosine. The average size of a representative preparation of viral DNA was shown to be 24S.

Attempts were made to separate viral and host DNAs from a mixture of both, by chromatography on methylated albumin columns. This proved unsuccessful, due probably to size heterogeneity of both DNAs under the conditions employed.

A method for the harvesting and lysis of Herpes-infected cells prior to RNA isolation was developed, and factors affecting the isolation of undegraded RNA from these infected cells were assessed; this resulted in the use of Bentonite at several points in the isolation schedule and the involvement of redistilled phenol at the appropriate stages.

The pattern of synthesis of RNA in BHK 21 (C13) cells was investigated over several short time periods. During a 30 minute pulse of radioactive precursor, RNA synthesised in these cells was consistently shown to comprise a 45S and a 32S component, with smaller amounts of synthesis of 18S and 4S RNA. Longer pulse times served to increase the incorporation into ribosomal RNA and sRNA. The existence of an RNA species of sedimentation coefficient greater than 45S was noted.

45S RNA was shown to be derived from the BHK 21 (C13) cell genome, and to be similar in base composition to 28S ribosomal RNA from these cells.

The observations presented here, together with analogous information from other laboratories, suggest that 45S (and probably also 32S) RNA contain ribosomal precursor material.

Infection of BHK 21 (C13) cells with Herpes Simplex virus gave rise to an almost immediate fall in overall RNA

synthesis which was correlated with the appearance of progeny virus in the same system. Fractionation of RNA synthesised during a 30 minute pulse experiment at several intervals after infection demonstrated that synthesis of 45S and 35S RNA diminished but remained host specific, and that an 18 to 23S RNA species was formed, maximally at 5 - 6 hours after infection. 4S RNA continued to be synthesised after infection

The 18 to 23S RNA was shown, in hybridisation experiments, to have been transcribed from the viral genome; a small fraction of this RNA hybridised specifically with host DNA also

Ribosomal RNA formed in infected cells was shown to be stable up to at least 6 hours after infection, while synthesis of ribosomal RNA in infected cells fell dramatically during this period. The S values of ribosomal RNAs isolated from BHK 21 (C13) cells were calculated to be 27S and 18S respectively; these values did not alter as a result of Herpes Simplex virus infection.

Addition of Mitomycin C to the growth medium suppressed the synthesis of RNA in BHK 21 (C13) cells before and after Herpes Simplex infection.

Theoretical considerations led to the conclusion that Herpes Simplex virus may code for the synthesis of a population of tRNA molecules in the infected cell. Exploratory experiments with partially purified and purified sRNA preparations, involving DNA : RNA hybridisation studies, demostrated that sRNA from BHK 21 (C13) cells was able to form a specific hybrid only with viral DNA and, to a certain extent, with host DNA.

An estimated 1.2% of the viral DNA was involved in sRNA coding, but attempts to quantitate the kinetics of production of the virus-specific sRNA were not fruitful. It was shown, however, that overall synthesis of sRNA did not diminish until 4 hours after infection.

Physical measurements on sRNAs from control and infected cells revealed no clear-cut differences between the two preparations. Formation in vitro of aminoacyl tRNAs using extracts of uninfected and infected cells demonstrated the presence of three arginyl tRNAs, three lysyl tRNAs, two seryl tRNAs and two prolyl tRNAs in BHK 21 (C13) cells. After infection with Herpes Simplex virus, a new arginyl tRNA and, possibly, a new seryl tRNA were elaborated.

Fractionation of T1 RNase digestion products of aminoacyl tRNAs confirmed and extended the significance of the above patterns of aminoacyl tRNA synthesis in BHK 21 (C13) cells.

The importance of the observations, in particular those relating to aminoacyl tRNA synthesis, were discussed with reference to current knowledge and speculation on the nature and functional organisation of the genetic code and the biological translation mechanisms which express it.

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CONTENTS.

Early definition of biological parameters		p. 1
SECTION 1. INTRODUCTION.		
1.	DEOXYRIBONUCLEIC ACID.	
1a	The Structure of DNA.	p. 6
1b	Unusual Bases in DNA	p. 7
1c	Tertiary Structure of DNA	p. 7
1d	Nucleotide Composition of DNA	p. 8
1e	Studies of DNA Base Sequence	p.10
1f	Shape and Size of DNA	p.11
1g	Behaviour of DNA in Solution	p.12
1h	Copolymer Formation	p.13
1i	Fractionation of DNA	p.13
2.	RIBONUCLEIC ACID.	
2a	Chemical Structure of RNA	p.15
2b	Transfer RNA	p.16
2c	Ribosomal RNA	p.18
2d	Messenger RNA	p.19
	Distribution	p.20
2e	Viral RNA	p.21
3.	DNA BIOSYNTHESIS.	
3a	General Considerations	p.22
3b	Activity *in vitro*	p.23
3c	*In vivo* Replication	p.24
3d	Mammalian Enzymes	p.26
3e	Physical properties	p.26
4.	RNA BIOSYNTHESIS	
4a	General Considerations	p.28
4b	Enzymes Involved	p.28
4c	*In vitro* Requirements	p.29
4d	Enzymes *in vivo*	p.30
4e	Mechanism of DNA Transcription	p.31
4f	Mechanism of RNA Duplication	p.31
4g	Kinetics of Synthesis	p.33
4h	Effect of Actinomycin D	p.34
5.	VIRUS STRUCTURE.	
5a	Coat Morphology	p.36
5b	DNA - Containing Animal Viruses	p.38
5c	Herpes Simplex Virus	p.38

6. BIOCHEMISTRY OF BACTERIOPHAGE T2 INFECTION.

6a Phage Structure and Function p.41
6b Biochemical Events p.41
6c Macromolecular Synthesis p.43

7. PROTEIN SYNTHESIS.

7a Ribosomes p.45
7b Messenger RNA p.46
7c Polysomes p.46
7d Transfer RNA p.47
7e The Code p.48

8. CONTROL OF MACROMOLECULAR SYNTHESIS.

8a Bacterial Systems p.50
8b Mammalian Cells p.51
The present Project p.54

SECTION II. EXPERIMENTAL.

MATERIALS p.56

METHODS.

I VIRUS GROWTH AND HARVESTING CONDITIONS

Ia Cell and Virus Growth p.58
Ib Harvesting of Cells and Virus p.58
Ic Differential Centrifugation p.60
Id Deoxyribonuclease and Ribonuclease Treatment p.60

2. PREPARATION AND USE OF FRACTIONATING COLUMNS.

2a Brushite Column p.60
2b Chromatography on Columns of MAK
(i) Preparation of Methylated Albumin (MA) p.61
(ii) Formation of MAK p.61
(iii) The MAK Column p.62
2c Fractionation on "Sephadex" G25 Columns p.63
2d Columns of DEAE-Cellulose p.63

3. ISOLATION OF DNA.

3a Method of Marmur p.64
3b The Method of Russell p.65
3c A Phenol Method (Siato & Miura) p.65
3d "Modified Marmur" Procedure p.66

4. Preparation of Bentonite p.66

5. PREPARATION OF RNA.

5a The Method of Kirby p.67
5b The Method of Fason, Cline and Smellie p.67
5c Preparation of sRNA p.68
5d Further Purification of sRNA p.69

6. QUANTITATIVE ESTIMATION PROCEDURES.

6a Estimation of Protein p.69
6b Estimation of DNA p.70
6c The Orcinol Method of Estimation of RNA p.70
6d Estimation of DNA and RNA using a Modification of the Schmidt-Thannhauser Technique p.70
6e Estimation of NaCl and CsCl p.71
6f Cell Fractionation Method p.71
6g Ribonuclease Assay p.72
6h Liquid Scintillation Counting p.72

7. FRACTIONATION OF RNA.

7a Treatment of Sucrose with Bentonite p.74
7b Sucrose Gradient Analysis p.74
7c Agarose Gel Electrophoresis p.75
7d Recovery of RNA From a Sucrose Gradient p.76
7e Hydrolysis of RNA p.76
7f Electrophoresis of RNA Nucleotides p.76

8. FRACTIONATION OF DNA.

8a Purification of CsCl p.77
8b CsCl Density Gradient Analysis p.78

9. HYBRIDISATION OF DNA WITH RNA.

9a Rapidly-Labelled RNA p.78
9b Soluble RNA p.79

10. PREPARATION OF AMINOACYL-tRNA SYNTHETASE.

10a Enzyme Purified on DEAE-Cellulose p.79
10b Crude Aminoacyl-tRNA Synthetase p.80

11. PREPARATION OF AMINOACYL tRNA.

11a Removal of Amino Acid from Aminoacyl tRNA p.80
11b Synthesis of Aminoacyl tRNA p.80
11c Treatment of Aminoacyl tRNA with T1 RNase p.82

SECTION III. RESULTS.
HERPES SIMPLEX DNA.

1. VIRUS PURIFICATION p.83

2. PREPARATION OF DNA p.84

3. CHARACTERISTICS OF ISOLATED DNA.

3a Biological Activity p.87
3b Buoyant Density of DNA in CsCl solutions p.88
3c Size of DNA product p.90
3d Melting temperature (Tm) determinations p.90
3e Complete separation of the two strands of DNA p.92
3f Effect of addition of salt to a solution of denatured Herpes Simplex virus DNA p.93

4. PREPARATION OF HERPES SIMPLEX VIRUS DNA. (Further considerations).

4a The influence of added protein and the use of Areton in preparation of DNA from Herpes Simplex virus p.94
4b Use of iso-propanol p.95

5. FRACTIONATION OF DNA.

5a MAK columns p.96
5b Caesium Chloride density gradient centrifugation p.99

RNA SYNTHESIS IN BHK21 (C13) CELLS BEFORE AND AFTER INFECTION WITH HERPES SIMPLEX VIRUS.

6. FRACTIONATION OF RNA.

6a Chromatography on MAK columns p.102
6b Sucrose Gradient Analysis p.103
6c Electrophoresis on Agarose Gel p.104

7. ASSESSMENT OF METHODS OF RNA FRACTIONATION p.105

8. ISOLATION OF RNA.

8a A comparison of the methods of Kirby and of Eason, Cline and Smellie p.105
8b Examination of Factors affecting the isolation of RNA p.106

8c Gel Filtration of isolated RNA p.109
8d Analysis of Sephadex-treated RNA on Sucrose gradients p.110
8e Assessment of methods of inhibition of RNase activity during RNA isolation p.111
8f Treatment of Sucrose with Bentonite p.112
8g Test fractionation of Krebs II Ascites Cell RNA and the use of Dialysis p.113

9. RNA SYNTHESIS AFTER HERPES INFECTION.

9a Effect on RNA synthesis of Infection of BHK21 (C13) cells with Herpes Simplex virus p.115
9b Effect of Mitomycin C. on synthesis of RNA p.116

10. PREPARATION OF RNA LABELLED WITH ^{32}P p.118

11. DNA-RNA HYBRIDISATION STUDIES.

11a Origin of 30 - 45S RNA from uninfected cells p.120
11b Origin of 30 - 45S RNA from infected cells p.122

12a RNA synthesis after infection-Extended time scale p.123
12b Origin of the 20S RNA formed after infection p.126

13. RIBOSOMAL RNA.

13a Effect of Herpes Simplex virus infection on ribosomal RNA synthesis p.129
13b Sedimentation Coefficient measurements on ribosomal RNA. p.130

14a Calculation of the S values of rapidly-labelled RNA p.131
14b Synthesis of labelled RNA in cells exposed to ^{3}H uridine for one to two hour periods p.132

15. TRANSFER RNA.

15a The genetic origin of "4S RNA" in Herpes Simplex infected cells p.136
15b Purification of infected cell sRNA p.137
15c Further DNA:sRNA Hybridisation Experiments p.139

16a Kinetics of Production of sRNA in Herpes Simplex virus-infected cells p.140
16b Examination of the stability of the DNA:sRNA hybrid to exhaustive RNase treatment p.142

17. PHYSICAL MEASUREMENTS ON sRNA.

17a Ultraviolet spectra p.143
17b Sedimentation coefficient Measurements p.144

17c Melting Temperature determinations p.145

18. An attempt to Hybridise infected cell sRNA with native BHK21 (Cl3) DNA and a bacterial DNA p.147

19. FORMATION OF AMINOACYL tRNA.

19a Preparation of ARSase and use of crude preparation p.149
19b Use of partially purified ARSase p.150
19c Isolation of aminoacyl tRNAs p.151
19d Stability of ARSase and aminoacyl tRNAs p.152

20. tRNA FORMATION USING A SINGLE LABELLED AMINO ACID.

20a Synthesis of Arginyl tRNA p.154
20b Repeat of MAK fractionation on the infection cell arginyl tRNA p.156
20c Investigation of the enzymes involved in the formation of arginyl tRNA p.156
20d ARSase specificity reinvestigated p.158

21a Formation of Seryl tRNA p.160
21b Formation of lysyl tRNA p.161

22a Formation of aminoacyl tRNA using unfractionated ARSase preparations p.162
22b Formation of prolyl-tRNA p.165

23. Digestion of aminoacyl tRNA using T1 RNase p.165

SECTION IV. DISCUSSION.

1. THE ANIMAL VIRUS GENOME.

Ia RNA- containing Animal Viruses p.169
Ib DNA - containing Viruses p.172

2. Herpes Simplex Virus DNA p.174

3. RNA Synthesis in Cultured Mammalian Cells p.177

4. RNA Synthesis in BHK21 (Cl3) Cells p.180

5. RNA SYNTHESIS IN VIRUS-INFECTED ANIMAL CELLS

5a RNA viruses p.182
5b DNA viruses p.183

6. The Infection of BHK21 (Cl3) Cells with Herpes Simplex Virus p.185

7. Alterations in Host-Specified RNA

Synthesis after Herpes Simplex Virus Infection p.187

8. SYNTHESIS OF TRANSFER RNA IN BHK21 (C13) CELLS BEFORE AND AFTER INFECTION WITH HERPES SIMPLEX VIRUS.

8a The uninfected cell p.188
8b Virus-specific synthesis p.191

9. Biochemical Events in the Herpes Simplex Virus-Infected Cell p.195

HORIZONS p.196

BIBLIOGRAPHY p.204

ABBREVIATIONS.

The following abbreviations will be used in this thesis:-

Tris	2-amino-2-hydroxymethylpropane-1,3-diol.
SSC	0.15M NaCl; 0.015M Sodium citrate, pH 7.0.
PPO	2,5-diphenyloxazole.
POPOP	1,4-di- 2-(5-phenyloxazolyl) -benzene.
A	Adenosine
G	Guanosine
C	Cytidine
U	Uridine
T	Thymidine
DEAE-cellulose	diethylaminoethyl cellulose.
MAK	Methylated Albumin bound to a supporting Kieselguhr matrix.
ATP	Adenosine 5' Triphosphate.
CTP	Cytidine 5' Triphosphate.
EDTA	Ethylenediaminotetra-acetic acid (Na Salt).
ligase	Aminoacyl sRNA synthetase (E.C.6.1.1.1. onwards).
RNase	Ribonuclease (E.C. 2.7.7.16).
DNase	Deoxyribonuclease (E.C.3.1.4.5.)
sRNA	soluble RNA
tRNA	transfer RNA (that fraction of sRNA which accepts amino acids).
mRNA	messenger RNA.
poly dX	3' - 5' polymer of deoxyribonucleotide X.

TMV	Tobacco Mosaic Virus
EMC virus	Encephalomyocarditis Virus.
WEE virus	Western Equine Encephalitis virus.
SV 40	Simian Virus 40.
c.p.m.	counts per minute.
d.p.m.	disintegrations per minute.
P.F.U.	Plaque-forming units.
m.w. units	molecular weight units.
SDS	Sodium Dodecyl Sulphate.
HSV	Herpes Simplex Virus.
MMV	Minute Mouse Virus.
I	Inosine
U^{h}	Dihydrouridine
χ	5-Ribosyluridine
I^{m}	Methyl Inosine

EARLY DEFINITION OF BIOLOGICAL PARAMETERS.

Disease, with the human suffering resulting from it, has been the incentive behind many fundamental biological discoveries.

(a) THE VIRUSES.

Diseases, by the middle of the 19th century, were known to be caused by at least two classes of harmful agent - the typical poisons or toxins, and, thanks to the work of Koch, Pasteur, Erlich and others the obviously living pathogenic microorganisms. It soon became evident, however, that there existed disease-causing agents which could be transmitted from host to host but which did not obey all the rules laid down by the bacteriologists for microorganisms. In particular, many of these agents were able to pass through filters capable of retaining bacteria: it was this property of infectious tobacco mosaic virus which led the Russian Iwanowski[i], unwittingly, to the first partial description of a virus[ii], although it was not until 1899 that Beijerinck, the Dutch Botanist, fully recognised this as a hitherto unknown type of biological organism: for this he coined the name "virus" - poison.

This first virus to be described multiplied as a parasite in a specific plant cell, but it soon became evident (Loffler & Frosch 1898)[iii] that animal cells also were able to act as hosts to virus particles (Foot and Mouth Disease Virus). Bacterial viruses were discovered by Twort[iv] in 1915 and described later by d'Herelle,[v] 1917, who first used the word bacteriophage, and who unsuccessfully attempted to discover therapeutic applications for these antibacterial microorganisms.

Two groups of pathogenic microorganisms are known which resemble bacteria (in that they have inherent metabolism) but which are only slightly larger than viruses; these are the rickettsiae and psittacosis group of organisms. Their relatively complex life cycles and metabolic activities exclude them from classification as viruses. (Schaechter, Bozeman & Smadel)[vi].

During the period 1920 - 1935, the new science of virology was of interest largely to biological research workers who were clinically orientated and consequently much work was carried out in the classification of virus diseases and in the discovery of new viruses.

W.M. Stanley[vii] in 1935 purified and subsequently crystallised TMV, which he believed at this time to be a protein: Bawden & Pirie,[viii] two years later, showed that

TMV was not just a protein but a nucleoprotein - 95% protein and 5% Nucleic Acid.

(b) NUCLEIC ACIDS.

The first description of a nucleic acid had been given by Miescher[ix] in 1868 who isolated nucleoprotein (he named it nuclein) from the nuclei of pus cells. Subsequently, he separated his nuclein into acidic (nucleic acid) and basic (protein) components: Altman[x] (1889) first described the preparation of protein-free nucleic acid from a variety of tissues.

By 1930, two nucleic acids were known - RNA and DNA. The Swedish chemists Hammarsten & Caspersson[xi] had shown that DNA could be prepared with a molecular weight of 5×10^5, but it was felt in view of the tetranucleotide hypothesis (widely accepted at that time but disproved by Chargaff[15] in 1947) that DNA was not the genetic material.

However, the subsequent discovery of nucleic acids in viruses and the successful experiments on pneumococcal transformation utilising isolated DNA (Avery et.al.[(1)]) proved that DNA was capable of carrying genetic information independently of any other chemical substance and that protein was not directly involved at this level.

(c) EARLY GENETICS.

In the same year that Miescher successfully isolated nucleoprotein crude cytological observations led Haeckel (1868)[xii] to propose that the cell nucleus was responsible for heredity in living systems: the cell theory of Schleiden & Schwann (1839)[xiii] had, by this time, gained general acceptance.

Proof, however, of the involvement of chromosomes in heredity and knowledge of the basic rules of heredity were not obtained until 1865, when Mendel[xiv] completed his brilliant series of experiments; these were ignored until 1900, and then confirmed by several laboratories, thus laying the foundation of modern genetics.

Almost immediately, geneticists began to speculate both on the chemical structure of the gene and on how it acts: it was realised then that nucleic acids and proteins are present in chromosomes, but neither were understood, either chemically or biologically.

Muller's[xv] discovery in 1927 of X-ray-induced mutations and Garrod's[xvi] proposal that a gene-enzyme relationship existed supported related work leading in the mid 1940's to the general acceptance of the hypothesis that genes control the synthesis of proteins, whether or not these are enzymes.

Thus biochemistry, genetics and virology converged at a time when it was being reasoned that viruses were no more "alive" than isolated chromosomes, to which they are very similar chemically and functionally. The resulting study of viruses and viral infection has proved to be of immense help in understanding how cells themselves live.

SECTION 1.

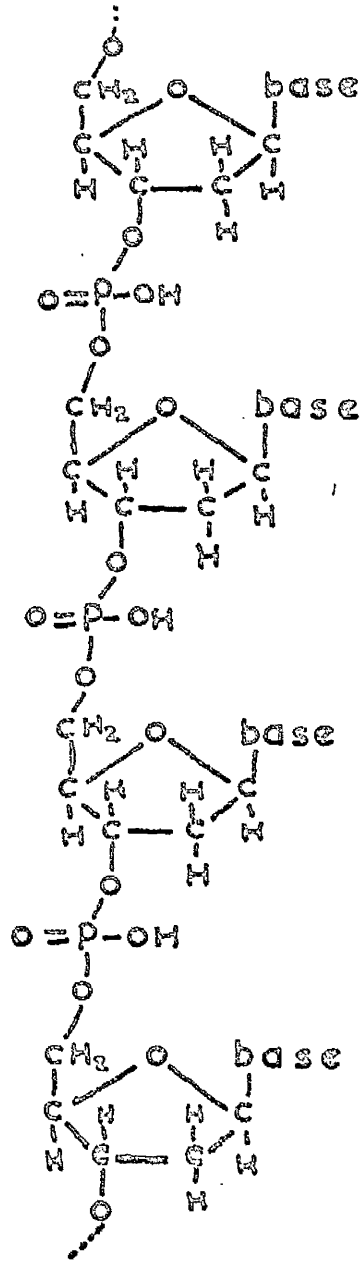
INTRODUCTION

F I G U R E 1.

A section of the polynucleotide chain in the DNA molecule.

(from Davidson, J.N., "The Biochemistry of the Nucleic Acids", 5th Ed., Methuen & Co., Ltd., London, 1965).

FIGURE 1



INTRODUCTION.

I. DEOXYRIBONUCLEIC ACID.

(a) The Structure of DNA.

DNA has been identified as the genetic substance (Avery et.al.; Beadle, G.W.; Weil,R.[1,2,3]), and with the possible exception of certain bacteria whose nuclear apparatus is less clearly defined, is almost completely confined to the nucleus, where it exists in combination with protein; DNA is also found in certain virus particles and in cytoplasmic particles such as chloroplasts (Chun et.al.[4]) and microchondria (Luck & Reich; Rabinowitz et.al. [5, 7]).

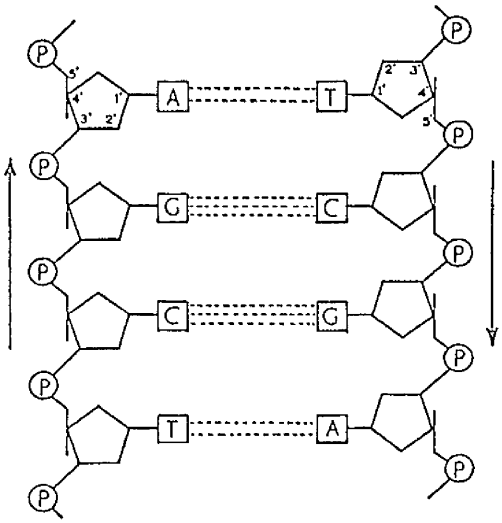
The chemical components of DNA have been deduced from extensive studies of chemical hydrolysis and enzymic degradation (Chargaff & Davidson [6]) and commonly consist of four bases, Adenine, Guanine, Cytosine, Thymine, the sugar β-2 deoxy D-ribose and phosphoric acid. The high molecular weight *in vivo* form of DNA involves non-random sequences of these bases bound to the C-1 position of deoxyribose; the deoxyribose molecules are, in turn, joined to a neighbouring sugar molecules via a 3' - 5' phosphodiester bond (Fig.1.) to give a linear unbranched polynucleotide.

F I G U R E 2.

Diagrammatic representation of part of a hypothetical polynucleotide chain in DNA.

(From Davidson, J.N., "The Biochemistry of the Nucleic Acids", 5th Ed., Methuen & Co., Ltd., London, 1965).

FIGURE 2



(b) Unusual Bases in DNA.

The common bases in DNA are, from time to time, replaced to a very small extent. 6-Methylaminopurine (Dunn & Smith [9]) and 5-Methylcytosine (Gold & Hurwitz [8]) can be isolated from several DNAs, while the bacteriophages T2, T4 and T6 contain 5-hydroxy methylcytosine instead of cytosine (Wyatt & Cohen[10]) and a glucose molecule bound to many of the hydroxy-groups (Lehman & Pratt[14]). 6-methylaminoadenine is a constituent of a thymine-less mutant of Escherichia coli (Smith[11]) and hydroxy methyl uracil and uracil respectively replace thymine in the DNAs of bacteriophages SP8 and PBSP 2 (Marmur & Cordes[12]); bacteriophages SP8 and a mutant SP8^{+}Ts derivative contain respectively D-glucose and D-mannose (Rosenberg[13]).

(c) Tertiary structure of DNA.

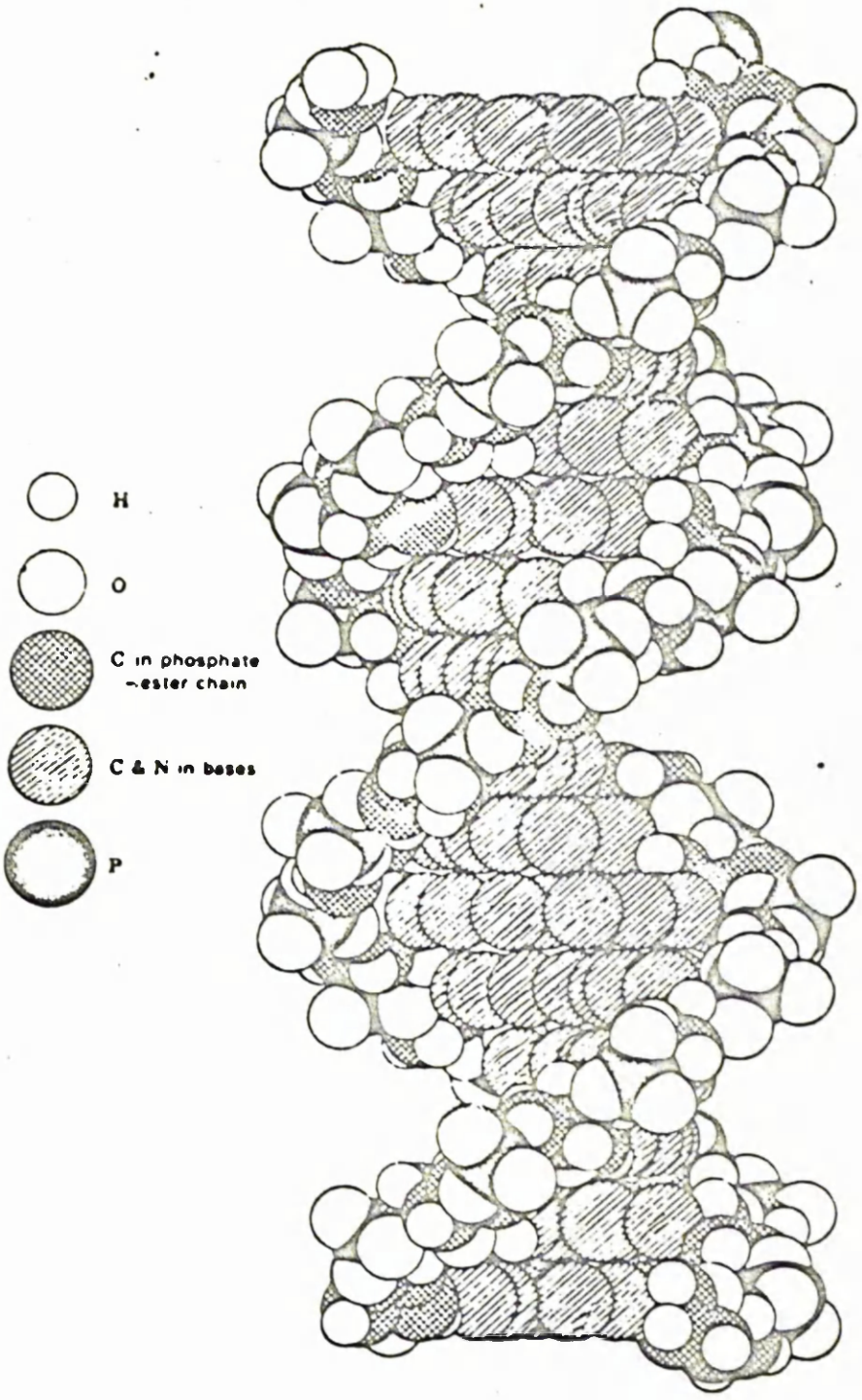
The work of Chargaff (Chargaff[15]) on the base composition of DNAs from many sources served to disprove the tetranucleotide hypothesis; concomitantly it was observed that the molar ratios of total purines to total pyrimidines, and also of A to T and of G to C were not significantly different from 1.

X-ray diffraction studies carried out on fibres of DNA by several groups (Astbury; Franklin & Gosling;

F I G U R E 3.

A model of the structure of DNA.
(after Feughelman, M., Langridge, R.,
Seeds, W.E., Stokes, A.R., Wilson, H.R.,
Hooper, C.W., Wilkins, M.H.F., Barclay, R.K.,
and Hamilton, L.D. (1955).
Nature, Lond., 175,834)

FIGURE 3



Wilkins [16, 17, 18]) indicated the presence of several chemical repeats of the phosphate sugar chain (Fig. 1.) in one structural repeat; these observations led Watson & Crick [19] to propose that the DNA molecule is a double right-handed helix consisting of two polynucleotide chains wound round the same axis and held together by hydrogen bonding between A and T and C and G (Fig. 2.) Fig. 3 represents a molecular model of the structure of DNA as corrected by Wilkins (1957) [20] and this offers a satisfactory explanation for many of its physical and biological properties.

(d) Nucleotide Composition of DNA.

Publications (see Kit [21] for review) initiated by Chargaff's original work (Chargaff [22]) have provided quantitative data on the nucleotide composition of DNA from species of every biological type. The mean G plus C content of DNA from bacteria varies from 25% to 75% (Davidson; Sueoka [23, 24]) in higher plants from 35% to 48% with a large amount of 5 methylcytosine present (Shapiro & Chargaff [25]), and in adult vertebrates from 40-44% (Kit [26]). The distribution of G plus C content of the DNA molecules within an organism is, with few exceptions (e.g. Joshi et.al.; Kit; [27, 29]) uni-modal

and of relatively narrow range: bacterial DNAs, in general, have much narrower distributions than those of higher organisms (Peacock & Drysdale [28]).

Many viruses contain small amounts of DNA, and many viral genomes are felt to consist of only one molecule of DNA. This DNA, particularly in the case of the bacteriophages, may have several unusual chemical properties e.g. bacteriophage α (Cordes et.al. [30]), which contains two DNA strands of unequal density. The DNA of a small number of bacteriophages is single-stranded (Sinsheimer [31]).

The base composition of the DNA of animal viruses follows a wide range of values, from 36% in vaccinia virus (Joklik [32]) to polyoma(50%)(Crawford [33]) and pseudorabies(74%)(Ben-Porat & Kaplan [34]). Herpes simplex virus has a G plus C content of 68% (Russell & Crawford [35]), although earlier work (Ben-Porat & Kaplan [34]) suggested that it was, in fact, even higher. There is no evidence for the occurrence of unusual bases or substituents in the DNA of animal viruses, and, with one exception, a minute virus of mice (Crawford [35a]), all appear to contain double stranded DNA: the DNA of Kilham rat virus may also be single-stranded (Whalley [36]).

(e) Studies of DNA Base Sequence.

While the nucleotide composition of many DNAs has been known for some time, methods do not at present exist for the elucidation of the exact sequence of nucleotides in a functional DNA molecule: nonetheless, Sonneborn [37] has published, on the basis of certain reasonable assumptions, the complete sequence of the gene in yeast corresponding to an alanyl transfer RNA. However, indirect evidence on the base sequence in DNA has come from such techniques as nearest neighbour frequency analysis (Josse et.al. [38]), in vitro DNA:DNA hybridisation studies (Schildkraut et.al. [39]) and examination of enzymic and chemical degradation products of DNA; this last technique has been useful in the examination of small fragments of DNA e.g. the discovery of pyrimidine oligonucleotide clusters in many DNAs (Spencer & Chargaff [40]). Molecular hybrid formation is valuable in assessing the genetic compatibility of DNAs from different sources but nearest neighbour study, which offers a statistical evaluation of DNA sequence for an entire genome, is the most informative method of sequence analysis at present. This has provided confirmation of the Watson-Crick model for DNA structure (Fig.3.) and, in common with the other techniques has

emphasised the non-random nature of DNA nucleotide sequence.

(f) Shape and Size of DNA.

DNA has been shown to be a highly extended, semi-rigid chain of asymmetric shape (Eigner & Doty [41]). The DNA genome of certain bacteria (Cairns [42]), viruses (Weil & Vinograd [43]), mammals and higher plants (Hotta & Bassel [44]) has been shown to possess a circular configuration. In polyoma virus, the circular genome exists in a twisted form (Vinograd et.al. [45]).

Although the DNA content of most bacterial cells corresponds to a molecular weight of several thousand million, most DNA preparations reported are in the size range 5-20 x 10^6 M.W. units (Sadron & Marmur [46]). This is a result of the susceptibility of DNA to degradation by hydrodynamic shear during isolation (Davison [47]). Recently, however, the preparation of a single DNA molecule comprising the entire genome of a virus (bacteriophage T2) (123 x 10^6 M.W. units) has been achieved (Rosenbloom & Schumaker [48]). Herpes simplex virus DNA has been prepared with a M.W. of 55 x 10^6 (Russell & Crawford [35]).

(g) Behaviour of DNA in Solution.

When solutions of DNA are exposed to elevated temperatures or extremes of pH, there is a collapse of the ordered secondary structure (Marmur et.al. [49]) called denaturation. The sharpness of this denaturation suggests a highly co-operative conformational change, compatible with the predicted behaviour of the DNA structural model (Fig. 3.) The molecule of denatured DNA, at ionic strengths of 0.1 - 0.2M or above folds back upon itself because of the formation of short, poorly organised regions of intra-strand base pairs: at lower ionic strengths the molecule becomes more extended in solution (Rownd [50]).

There is now little doubt that absolute denaturation involves the complete separation of the two strands of native DNA (Rownd & Doty [51]). Thermal denaturation of DNA under controlled conditions is a valuable analytical procedure: the thermal stability of a given DNA varies with its G plus C content (Doty et.al. [52]) and the ionic strength of the solvent (Dove & Davidson [53]).

Denatured DNA may be renatured under certain conditions to form a population of molecules whose buoyant densities in CsCl range from that of the denatured DNA to values about as low as the native density

(Rownd et.al.[54]), and whose transforming properties are not completely lost (Marmur & Lane[55]). The transforming ability of DNA may be destroyed by treatment with DNase, ionising radiation, reaction with various chemical agents e.g. nitrous acid (Tessman[302]), or decay of incorporated ^{32}P, none of which need necessarily involve extensive breakdown of DNA secondary structure (Ravin [56]).

(h) Copolymer Formation.

The formation *in vitro* by a bacterial enzyme under unusual conditions of alternating copolymers e.g. polydAT and homopolymes e.g. polydGdC has been described (Radding et.al.[57]). The immediate *in vivo* significance of such synthesis is difficult to assess, but a minor component of crab DNA has been described (Sueoka & Chong [58]) with a G plus C content of 2 - 3% only.

(i) Fractionation of DNA.

Several methods exist for the fractionation of DNA molecules. Columns of methylated albumin bound to Kieselguhr separate DNAs (Sueoka & Chong[59]) on the basis of (i) base composition; (ii) molecular weight; (iii) and whether they are single or double-stranded. Columns of ECTEOLA cellulose fractionate on the basis of the first two properties of the DNA (Kit[59]). Zone electrophoresis on

starch has been used in large scale separation of native DNA from denatured DNA and RNA (Matsubara & Takagi [60]). The buoyant density of a DNA molecule in density gradients of CsCl or Cs_2So_4 (Meselson et.al. [61]) depends on its nucleotide composition and strandedness, and this property has been used preparatively and analytically in a wide variety of studies. Recently (Walker & McLaren [62]) mammalian DNA samples have been examined on columns of hydroxylapatite, and some heterogeneicity of composition has been noted.

II. RIBONUCLEIC ACID.

(a) Chemical Structure of RNA.

RNA is a polynucleotide similar in many respects to DNA: the molecule consists of a linear order of repeating mononucleotides mutually bound by 3' : 5' - phosphodiester bonds in a fashion analogous to DNA. The major differences between these two polymers occur inside the mononucleotide unit in that the pentose in RNA is β-D-ribose and the commonly occurring bases are Adenine, Guanine, Cytosine and Uracil. Several unusual bases whose intra-molecular distributions are non-random (Staehelin [79]) have been isolated from RNA in a wide range of species. Transfer RNA is particularly rich in these bases, containing up to 4% 5-ribofuranosyl uracil (Cohn [64]), a variety of methylated nucleosides, chiefly Thymidine, 5-methylcytidine, 1-methyladenosine, 2-methyladenosine, 6-methylaminopurine nucleoside, 6-dimethylaminopurine nucleoside, 1-methylguanosine, 6-hydroxy 2-methylaminopurine nucleoside and 6-hydroxy dimethylaminopurine nucleoside (Dunn et.al. [65]). Recently the presence of 5,6-dihydrouridylic acid has been detected in yeast tRNA (Madison & Holley [66]). Using a methionine-requiring Escherichia coli auxotroph Starr and Fefferman have provided evidence (Starr & Fefferman [67]) for the in vivo methylation of

ribosomal RNA. The results suggest that the distribution of methylated purines and pyrimidines in this RNA fraction is similar to that in transfer RNA. Enzymes involved in the formation of these methylated bases have been isolated and will be discussed later.

RNA occurs in the cytoplasm and, to a small extent, in the nucleolus of all cells (Davidson [63]) and may be classified into several types - amino acyl transfer RNA; ribosomal RNA; viral RNA, and messenger RNA.

These four types of RNA all have the same covalent primary structure but their tertiary structural properties differ distinctly.

(b) Transfer RNA.

The existence of a soluble RNA species with amino acid transfer properties is well established (Hoagland [68]). Nucleotide analyses of mixed transfer RNA molecules from yeast, bacteria or mammalian organisms indicate that the amounts of guanine and adenine residues are equal to the cytosine and uracil residues respectively (Allen et.al. [69]). This indicates a common structure, base-paired according to the Watson-Crick scheme.

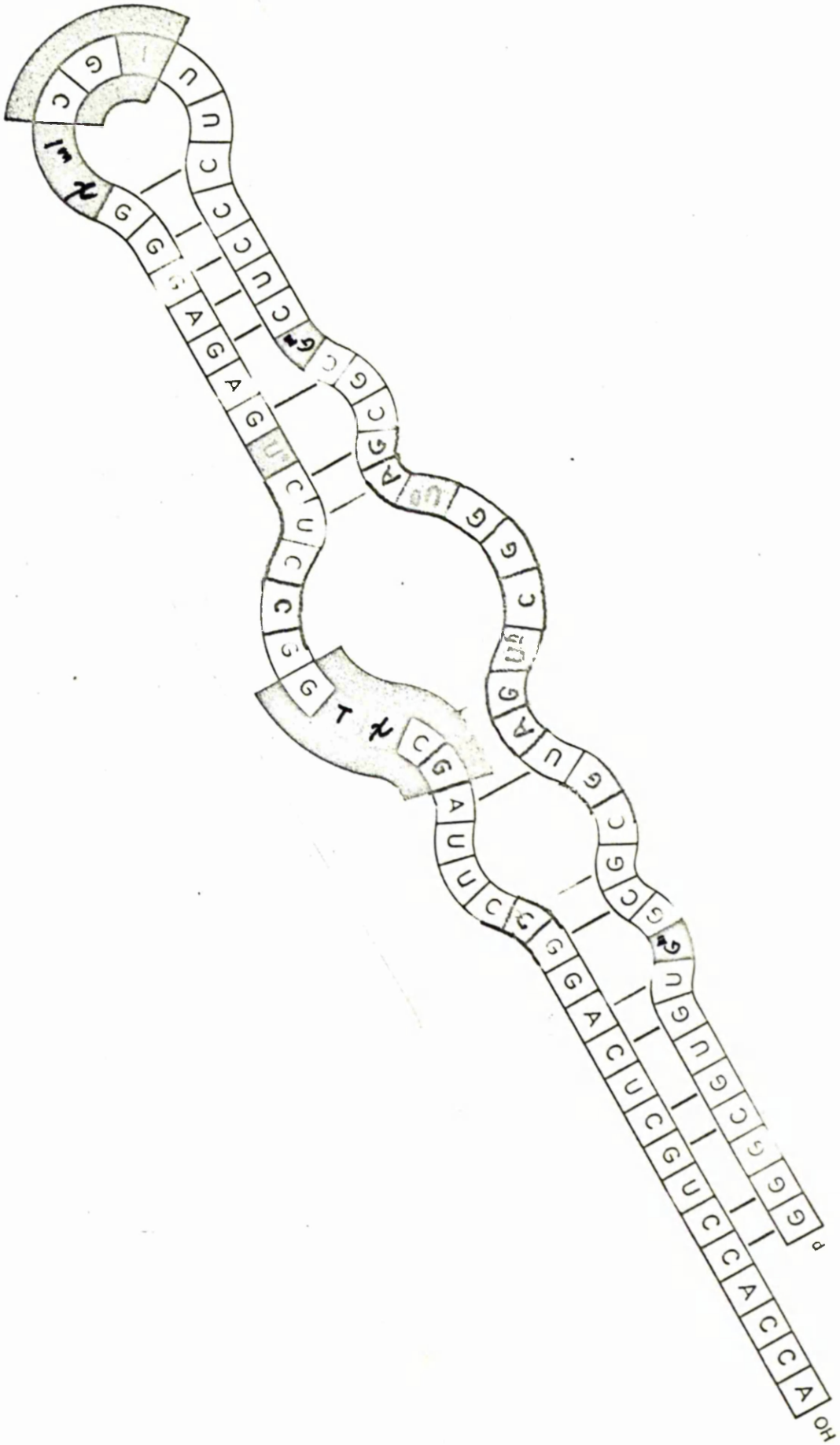
The molecular weight of t-RNAs from different species is relatively uniform: values of 24,000 ± 2,000 M.W.

F I G U R E 4.

The Nucleotide sequence and a possible structure of a yeast alanyl tRNA.

(From Holley, R.W.(1966). Sci.Amer., 214(2),30.)

FIGURE 4



units have been reported for Escherichia coli, liver and yeast tRNA (e.g. Osawa [70]). Moreover, in rabbit-liver, the sedimentation coefficients of sRNA chains labelled with [^{14}C]leucine, proline or valine did not differ significantly among themselves or in relation to the preparation as a whole (Kloe & Cantoni [71]). At present, owing to lack of refinement of physical methods it cannot be established whether all tRNA molecules contain the same number of nucleotides (Brown [72]).

However, it appears that each molecule consists of one polynucleotide chain (Brown & Zubay [73]), the 3' hydroxy terminal sequence of which is pCpCpA, (Preiss, et.al. [74]). At the 5' terminal end, the base is usually guanine (Singer & Cantoni [75]).

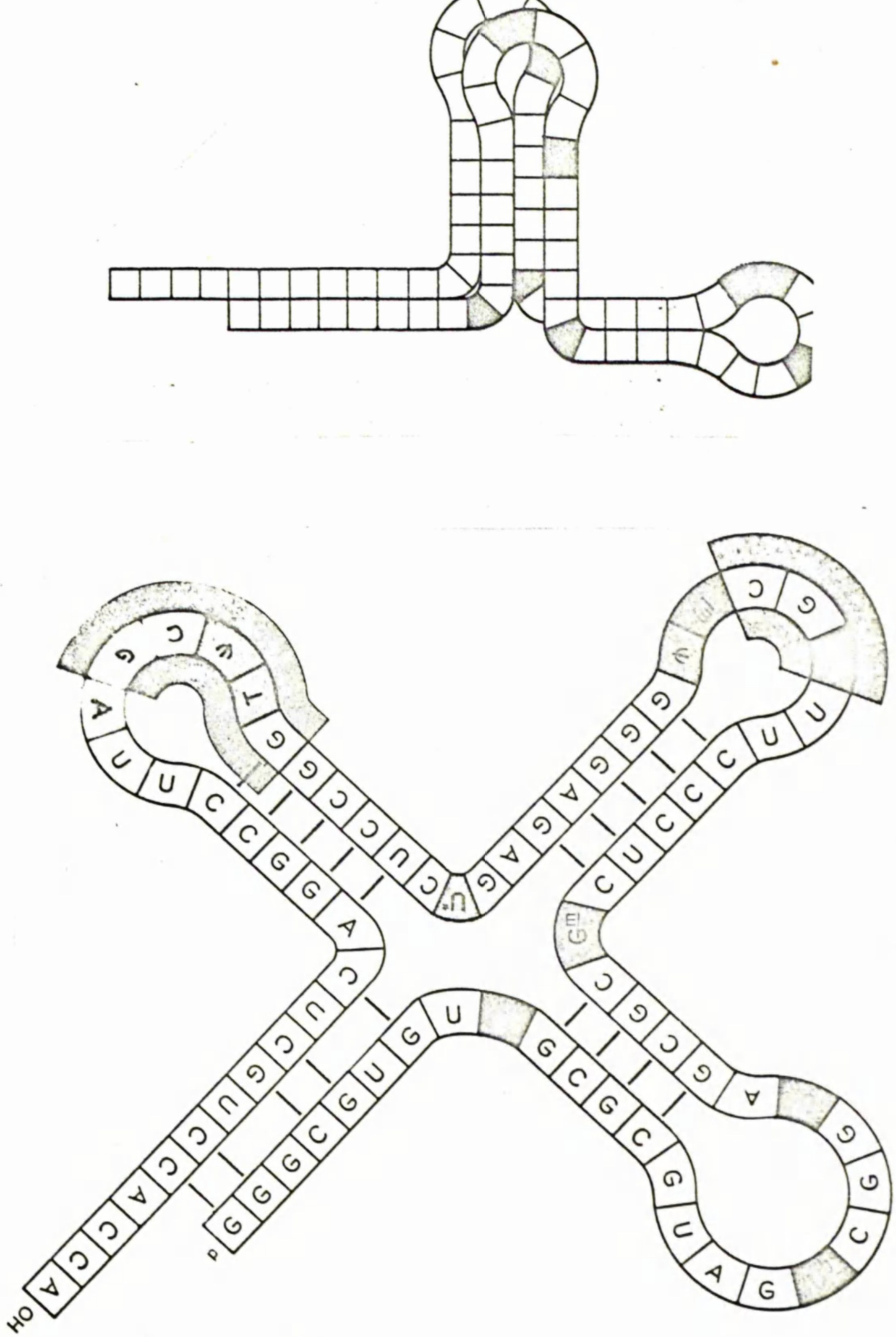
Holley and his collaborators (Holley et.al. [76]) have recently isolated from yeast, by counter current distribution techniques, a pure alanyl transfer RNA. Analysis of this fraction has resulted in the complete characterisation of the base composition and sequence of this tRNA molecule (Fig. 4.). This analysis has largely confirmed the theories based on less critical examinations of impure tRNA samples, but unfortunately has not led to an elucidation of the tertiary conformation of the molecule. It had already been

F I G U R E 5.

Two additional structural possibilities for a yeast alanyl tRNA.

(from Holley, R.W. (1966). Sci. Amer., 214(2), 30.)

FIGURE 5



suggested by X-ray diffraction studies (Spencer et.al. [77]) that tRNA contained regions of Watson-Crick pairing, and hair-pin like models were constructed on this basis (Spencer [78]). Models constructed as a result of more recent work are shown in Fig. 5.

Transfer RNA, in accordance with its suggested secondary structure exhibits an extended hyperchromic effect in solution as the temperature is raised (Staehelin [79]). The temperature absorbancy profile may be sharpened considerably in the presence of Mg^{2+} (Monier & Grunberg-Manago [80]) and small differences in Tm exist between specific tRNAs.

(c) Ribosomal RNA, the major constituent of the cell's complement of RNA, can be separated without difficulty from tRNA. The major factor in this separation (Britten et.al. [80]) is the molecular weight difference between ribosomal RNA and tRNA: ribosomal RNA occurs in bacteria, mammals and plants as two moeties, ranging in size from 16S and 23S (bacteria, (Hall & Doty [81]) to 18S and 32S (mammalian cells, (Timasheff et.al. [82]) and corresponding to molecular weights of 5×10^5 and 10^6 respectively. In vivo these molecules occur in combination with protein as respectively 30S and 50S

particles, which together form a ribosome (Kurland [83]).

Base composition analysis of ribosomal RNAs has shown (Spirin [84]) that there is no overall equality between G and C or A and U, and that no correlation with the DNA of the same organism can be made on this basis. The results, taken together with the physical behaviour of ribosomal RNA in solution (Spirin [85]) have led to the structural delineation of ribosomal RNA as a single polynucleotide chain containing helical regions involving hairpin turns of the chain and conventional A-U, G-C base pairing (Fresco et.al. [86]), as suggested for tRNA (Fig. 5.). On the basis of studies on the hyperchromic effect observed, it has been suggested (Schlessinger [87]) that the helical configuration of ribosomal RNA is essentially retained when the ribosomal RNA is functional in a ribosome.

(d) Messenger RNA.

The molecular characteristics of messenger RNA have not been established, but it seems reasonable that secondary structure may be deliberately poorly developed and of no consequence to its function. Volkin & Astrachan [88] first proposed the existence in bacteriophage T2 infected Escherichia coli of an unstable RNA fraction

with base ratios identical to the bacteriophage DNA: this work was supported by an examination of the regulating systems in bacteria (Jacob & Monod [89]) and the RNA was named messenger RNA (mRNA). It has been proposed that mRNA should be kinetically unstable; have a composition mimicking that of the DNA of the cell and be able to act as template for protein synthesis (Roberts [90]).

Several values for the size of mRNA in uninfected and virus-infected bacterial cells have been obtained, from 8S in bacteriophage T2 infected Escherichia coli (Nomura et.al. [91]) to 23-30S (Ishihama et.al. [92]) in Escherichia coli. mRNA has a half-life of 2 minutes in Bacillus subtilis (Levinthal et.al. [93]), but nevertheless is able to function many times as a template for protein synthesis.

(b) Distribution.

A similar fraction of RNA with the DNA-like composition and instability of mRNA has been shown in yeast cells (Yeas & Vincent [94]) and pea seedlings (Korner & Munro [95]). Rapidly-labelled RNA from mammalian cells has a much longer life (8-12 hours in the rat (Korner & Munro [95]) and 3-4 hours in Hela cells (Penman et.al. [96]), especially in the reticulocyte

(at least 6 hours). (Marks et.al.[97]). This mammalian rapidly-labelled RNA mirrors DNA in its base composition and generally satisfies the criteria laid down for bacterial messenger RNA (Hill et.al.[98]) except that no evidence of its direction of specific protein synthesis has been presented; its existence is therefore questioned (Harris [99]). An mRNA fraction has been partially characterised in vaccinia (DNA virus) infected Hela cells (Becker & Joklik [100]); this RNA fraction is not found in uninfected cells nor is it a constituent of the infective virus particle. It is, however, transcribed from the virus genome.

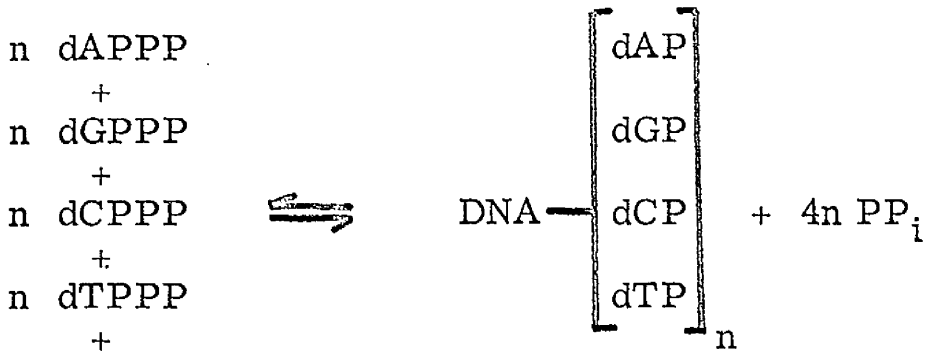
(c) Viral RNA.

The RNA component in both single and double-stranded RNA viruses has been shown to have a messenger RNA function, and may be regarded as a stable mRNA.

F I G U R E 6.

The mechanism of DNA biosynthesis.
(after Lehman, I.R., Bessman, M.J., Simms, E.S.
and Kornberg, A. (1958). J.biol.chem.,
233, 163).

FIGURE 6


n dAPPP
+
n dGPPP
+
n dCPPP
+
n dTPPP
+
DNA primer
dAP
dGP
dCP
dTP
n
DNA
+ 4n PPi

III. DNA BIOSYNTHESIS.

(a) General considerations.

The double-helical structure of DNA allows the possibility of self-duplication; the mechanism of replication of DNA in vivo must also involve specificity and allow for modification and variation of the genetic material.

DNA nucleotidyltransferase (E.C. 2.7.7.7.) will, in the presence of a divalent cation, the four deoxyribonucleoside triphosphates and "primer" DNA catalyse the formation of a complementary copy of the "primer" (Fig. 6.). In bacteria, the enzyme is associated with DNA (Dillon [102]), but in mammalian cells enzyme activity may be found in both nucleus and cytoplasm (Keir et.al. [103]) apparently with identical properties (Hay [104]). This distribution has been correlated with DNA synthesis (Littlefield et.al. [105]) in L-cells. DNA synthesis takes place in mammalian cells during a characteristic part of interphase, the S phase (Watson [106]) while in bacteria, synthesis occupies a variable part (normally about 50%) of the generation time (Maaløe [107]).

(b) Activity in vitro.

DNA nucleotidyltransferase has been purified in several bacterial systems (Zimmerman & Goldschek[108]); the enzyme from Bacillus subtilis has been obtained free of exo-and endonuclease activities (Zimmerman & Goldschek[108]) while Escherichia coli DNA nucleotidyltransferase appears not to be physically separable from an exonuclease activity (Zimmerman & Goldschek[108]). These two enzymes, under certain conditions, will utilise both double and single-stranded DNA as templates (Zimmerman & Goldschek: Kornberg: [108, 109]) as will DNA nucleotidyltransferase from HSV-infected BHK21 (C13) cells (Keir et.al.[112]) and from sea-urchin embryos (Mazia[113]).

Generally, however, single-stranded DNA is a more effective primer for DNA nucleotidyltransferase in vitro than native DNA: this has been demonstrated for mammalian as well as bacterial and phage-induced DNA nucleotidyltransferase (Bollum[110]). Keir (1965) (Keir [111]) has interpreted the action of these DNA nucleotidyltransferases from diverse organisms by postulating the existence of an "intact form" of DNA nucleotidyltransferase able to use native DNA, and an "altered form" active only in the presence of single-

stranded DNA.

(c) In Vivo Replication.

In 1957, Levinthal and Thomas (Levinthal & Thomas [113]), using bacteriophage T2 DNA, gave physical evidence for a complementary semi-conservative system of DNA replication: more direct support for this mechanism in Escherichia coli derives from the work of Meselson and Stahl (Meselson & Stahl [114]). Similar observations, using Bromodeoxyuridine incorporation, on the replication of mammalian (Chun & Littlefield [115]) DNA have been made: Chlamydomonas DNA replicates in this way (Meselson & Weigle [116]). Cavalieri and Rosenberg (Cavalieri & Rosenberg [117]) regard the double helix of DNA as the conserved unit, and not the single polynucleotide chain: however bacteriophage DNA, which exists as one molecule, has been found in a hybrid form (Meselson & Weigle [116]) and theory of Cavalieri and Rosenberg is not generally accepted.

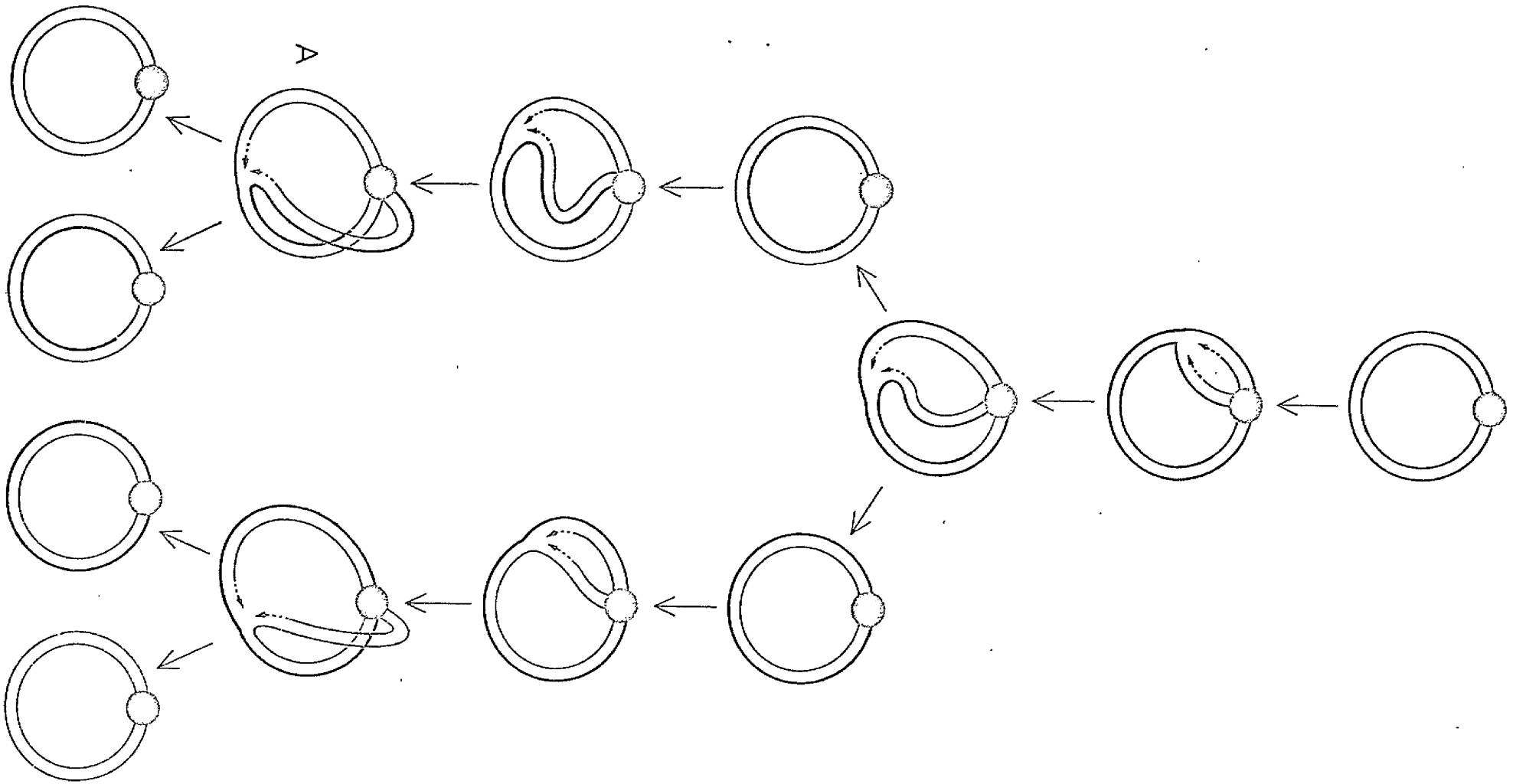
The mechanism of replication of a single-stranded oligodeoxynucleotide is not difficult to visualise in terms of the Watson-Crick scheme: however, in vivo, DNA exists as a double helix, and much evidence has accumulated for the existence of circular molecules

F I G U R E 7.

Schematic representation of the replication of a circular bacterial DNA molecule.

(from Cairns, J., (1966). Sci. Amer., 214(1), 36).

FIGURE 7



of DNA in a variety of systems (Elson [118]).

The process of replication of a double-stranded circular DNA has been summarised by Cairns (Cairns [119]) using Escherichia coli. This is represented in Fig. 7. Conclusions compatible with Cairns' model have been arrived at by(Yoshikawa & Sueoka [120]) in Bacillus subtilis using a genetical approach. This model for the action of DNA nucleotidyltransferase implies that the enzyme, moving in one direction, synthesises two polynucleotide strands of opposite polarity: this may involve the use of 3' deoxynucleoside triphosphates as well as 5' deoxynucleoside triphosphates and must involve two different enzyme specificities, if not two enzyme molecules. Several hypotheses may explain this apparent paradox, e.g. (Sibatani & Hiai [121]). These models have not been worked out for higher organisms, but the situation there may be similar.

An elegant control mechanism for replication in bacteria has been proposed (Jacob & Brenner [122]) involving a unit of replication (<u>replicon</u>) and two specific determinants: (a) a structural gene controlling an <u>initiator</u> acting upon (b) a <u>replicator.</u>

(d) Mammalian Enzymes.

DNA nucleotidyltransferase has been purified substantially free from nuclease activity in only two mammalian systems (Shepherd & Keir: Yoneda & Bollum [123], [124]). Bollum, using calf thymus gland, has also purified terminal DNA nucleotidyltransferase, an enzyme which, in the presence of one triphosphate, will add this to the end group of a DNA single strand (Krakow et.al. [125]). The purified replicative enzyme will not use native DNA nor will it associate with DNA in a molecular complex (Yoneda & Bollum [124]).

Recently, it has been demonstrated that herpes simplex virus induces a new DNA nucleotidyltransferase in hamster kidney cells (Keir et.al. [126]). This enzyme activity is predominantly nuclear in origin (Keir & Gold [127]) and, unlike the host cell enzyme, is capable of utilising both single and double stranded DNA as primer (Morrison & Keir [128]).

(e) Physical Properties.

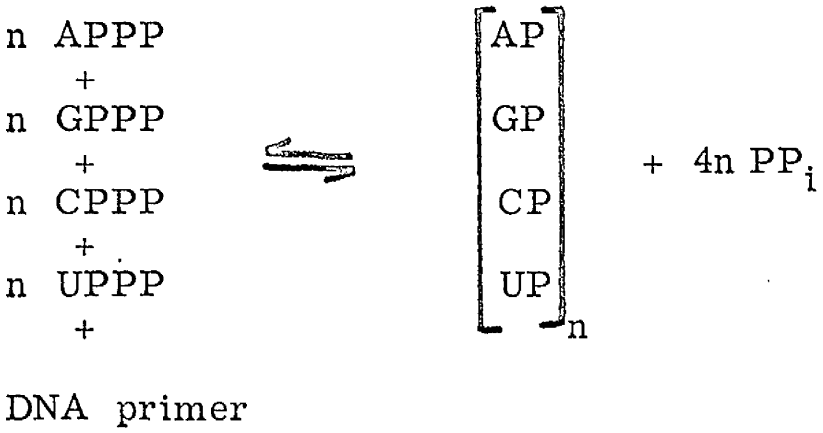
The Escherichia coli DNA nucleotidyltransferase has been shown by Baldwin (Baldwin[129]) to have a M.W. of 10^5 (5.6S), while the calf thymus replicative DNA nucleotidyltransferase has a S coefficient of 12.2.

(Yonoda & Bollum [124]); a molecular weight of about 10^5 is suggested by gel filtration studies (Shepherd[394]). Both enzyme activities are unstable, especially the mammalian one, and this remains a hazard in further purification.

F I G U R E 8.

The mechanism of RNA biosynthesis on a DNA template.

FIGURE 8



IV. RNA BIOSYNTHESIS.

(a) General Considerations.

In 1955 Ochoa et.al. (Grunberg-Manago et al.[141]) isolated from A.Vinelandii cells an enzyme which, in the presence of the four ribonucleoside diphosphates, was able to synthesise an RNA product. At that time, this enzyme was felt to be responsible for the synthesis in vivo of RNA: this view is not now held, and the existence of enzymes which catalyse the formation of RNA from ribonucleoside 5' triphosphates in the presence of primer DNA is well established in a variety of organisms (Weiss[143]).

(b) Enzymes Involved.

That this enzyme, RNA nucleotidyltransferase (2.7.7.6) has been observed in every living organism examined is not surprising: the general reaction catalysed is shown in Fig.8.

RNA nucleotidyltransferases, are readily isolated complexed with DNA, (e.g. Kadoya et.al.[144]) and the active enzyme may readily be obtained from several DNA-containing structures in the mammalian (Gorski[145]) and plant cell (Chipchase et.al.[146]) and from the nuclear region in bacteria (Oishi et.al.[147]).

It has been shown in only one case that RNA nucleotidyltransferase activity is increased in cells infected with a DNA-virus (Russell et.al. [148]); in contrast a great deal of work has resulted in the characterisation of enzymes induced in RNA virus-infected cells which synthesise RNA from the four nucleoside triphosphates e.g. (Horton et.al. [149]). The RNA virus-induced enzyme will use as template for RNA synthesis the infecting viral RNA, be it single or double stranded: this distinction between DNA and RNA-primed RNA nucleotidyltransferase on the basis of an *in vivo* template involvement may not be a relevant one *in vitro*.

(c) In Vitro Requirements.

The most highly-purified RNA nucleotidyltransferases, those from *Escherichia coli* and *Micrococcus lysodeikticus* (Gomatos et.al.[150]) exhibit a remarkable versatility *in vitro* with respect to primer requirements (Elson [151]), using one or two-stranded DNA or RNA or the corresponding synthetic polynucleotides, faithfully forming complementary copies. However, some RNA nucleotidyl-transferases do, in fact, exhibit a restricted primer requirement, and it may well be that such specificity as

did reside with, e.g. the Escherichia coli enzyme in vivo, has been lost in purification. It is important to note that an RNA-stimulated nucleotidyltransferase of Escherichia coli has been characterised as distinct from the DNA-primed enzyme (August et.al.[152]) and that the DNA-primed RNA nucleotidyltransferase for the same organism apparently exists as a hexamer.

(d) Enzymes in vivo.

The action of RNA nucleotidyltransferase (E.C.2.7.7.6) in vivo appears to be specific, and evidence from both bacterial and mammalian cells indicates that all types of cell RNA are derived ultimately from DNA (See section g). In vitro, RNA nucleotidyltransferase copies both strands of a double-stranded primer (Weiss & Nakamoto[153]) but, as a result of several pieces of evidence, it is felt that this does not occur in vivo. Asymmetric transcription in vivo has been inferred from work in bacteriophage (Fox & Meselson[154]), Pneumococcus (Guild & Robison[155]), Escherichia coli (McCarthy & Bolton[156]) bacteriophage SP8 (Marmur & Greenspan[157]), but not so far, in a mammalian system: the work of Dresler et.al. on Bacillus subtilis (Dresler et.al.[158]) is not in agreement with

this hypothesis.

(e) Mechanism of DNA transcription.

In attempts to characterise the molecular basis of the specificity of transcription *in vivo* two groups of workers (Hayashi *et.al.*: Goldschek *et.al.*[159,160]) have succeeded in obtaining asymmetric transcription *in vitro* using bacterial enzymes, but [illegible] came to different conclusions. Hayashi et.al., using the purified *Escherichia coli* RNA nucleotidyltransferase found that only the intact circular replicative form of bacteriophage ϕX 174 DNA is transcribed asymmetrically, while Goldschek *et.al.*, suggest that the enzyme is the controlling factor: they were able to retain asymmetry of transcription from any double-stranded DNA template using a crude enzyme from *Bacillus megatherium*, but not using a purified preparation of RNA nucleotidyltransferase. Both parameters may well be involved in preservation of asymmetrical transcription in this case.

(f) Mechanism of RNA duplication.

Infection of a cell by a double-stranded RNA virus gives rise to the production of virus-induced RNA nucleotidyltransferase, which replicates the RNA semi-

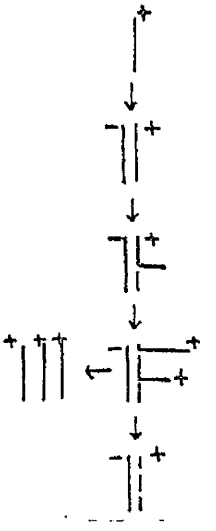
F I G U R E 9.

a. Diagrammatic representation of the replicating mechanism of a single-stranded virus RNA.
(after Weissman, C., Borst, P., Burdon, R.H., Billeter, M.A., and Ochoa, S. (1964). Proc. nat. Acad. Sci., Wash., 51, 682.

b. The replication scheme for single-stranded viral RNA as proposed by Brown, F. and Martin, S.J. (1965).
Nature, Lond., 208, 861.

FIGURE 9

(a)



Ingoing RNA (1)

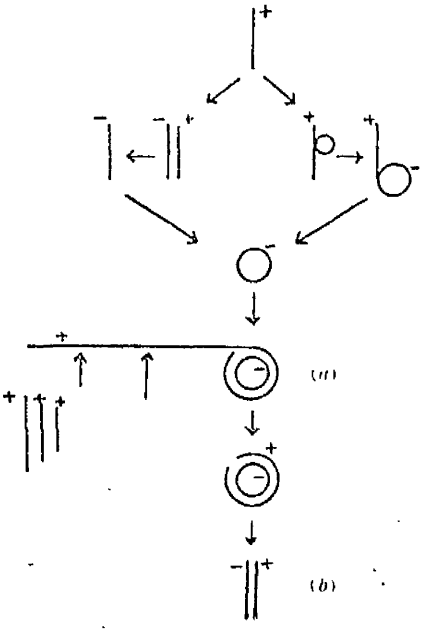
Double-stranded RNA formed (2)

Original plus strand is displaced by a new plus strand (3)

(*a*) Preferential synthesis of plus strands of equal length occurs (4)

(*b*) Ribonuclease treatment degrades the 'tails' and yields an incomplete double-strand (5)

(b)



Ingoing RNA (1)

Minus strand synthesized possibly as a ring (2)

The RNA polymerase can now rotate round the circle and produce a long chain of plus strand (3)

(*a*)

RNase treatment degrades the side chain and opens the ring yielding a complete double strand (4)

(*b*)

conservatively in a fashion analagous to DNA replication (Comatos & Tamm[139]). Few RNA viruses are double-stranded, however, and several groups have proposed schemes for the replication of single-stranded viral RNA using the induced enzyme.

Double-stranded RNA has been found in small amounts in preparations from Encephalomyocarditis virus-infected cells (Montagnier & Sanders[162]); since then it has also been reported in certain plant virus-infected and bacteriophage-infected cells (Weissman et.al.[164]). On the basis of these findings, it was suggested that the double-stranded RNA was an obligatory intermediate in replication of the viral genome, and a model of replication was constructed (Fig.9a). Fig.9b shows a similar model modified from the Ochoa concept to explain some inconsistencies in the product RNA from a system infected by Foot and Mouth Disease Virus (Brown & Martin[165]). The very recent work of Haruna and Spiegelman, however, on bacteriophage Qβ and MS2 (Haruna & Spiegelman[166]) infection of Escherichia coli, from which they isolated and purified an RNA dependent RNA polymerase (replicase) with an absolute requirement for the intact single-stranded RNA of the parent virus, is not consistent with the above earlier work. These

authors invoke a non-complementary base pairing model of replication, in which no double-stranded intermediate is necessary. This type of base-pairing is chemically feasible.

(g) Kinetics of Synthesis.

In uninfected cells, RNA synthesis takes place in predominantly the nucleus (Prescott [168]) and, at least in some cells, all of this RNA is transferred to the cytoplasm; in general, however, it appears that some RNA may be retained in the nucleus. Harris (Harris [169]) has suggested a mechanism for the breakdown in the nucleus of rapidly labelled RNA, which is certainly broken down in the cytoplasm to a large extent. (Taylor [169]).

RNA synthesis is continuous in the nucleus throughout interphase, but ceases during mitosis, probably because of chromosome condensation (Prescott & Bender [170]). DNA and RNA synthesis from a DNA template are mutually exclusive (Prescott & Kimball [171]).

Evidence from DNA-RNA hybridisation experiments suggest (Hall & Spiegelman [172]) that transfer RNA (Goodman et.al. [173]) the ribosomal RNAs (Yankofsky & Spiegelman [174]) and the many messenger RNAs each arise from separate loci on the DNA template.

Ribosomal RNA is synthesised from loci clustered in the nucleolar organiser region of Drosophila melanogaster (Ritossa & Spiegelman[175]) and evidence for the involvement of the nucleolus in ribosomal RNA synthesis in other systems is strong, e.g. (Birnstiel et.al.[176]).

Transfer RNA is synthesised in the nucleus (Perry [177]) as would be predicted from its DNA origin, and this synthesis, along with ribosomal and messenger RNA synthesis is sensitive to the antibiotic actinomycin D (Reich et.al.[178]), as is the transcription of DNA in vitro. DNA replication in vitro is sensitive only to higher levels of the antibiotic (Kelr et.al.[178]).

(b) Effect of Actinomycin D.

The turnover of nucleotides at the 3' terminal of tRNA is not sensitive to Actinomycin D (Reich et.al.[178]), nor is the synthesis of RNA from RNA templates (Cline et.al.[179]). No such synthesis has been detected to date (Smellie [180]) outside a virus-infected cell system. (Laszlo et.al.[130]) have shown that the effect of Actinomycin D is complex and not confined to Nucleic Acid metabolism.

Sedimentation characteristics of rapidly-labelled RNA have been examined by several groups working with

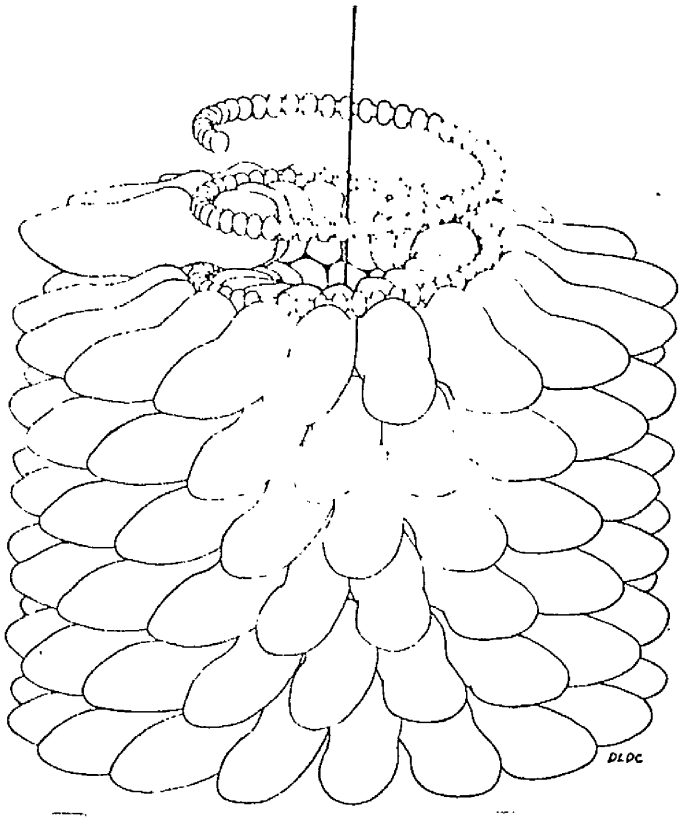
mammalian cells (Scherrer & Darnell[181]). In the initial stages of synthesis labelled precursor is incorporated into material of 50 - 35S; the latter then breaks down, not qquantitatively, to the two ribosomal RNA species. Transfer RNA is present continuously. The identity of messenger RNA in this system (Hela cells) is obscure, but (Scherrer et.al.[182]) have found "messenger" RNA activity at 45S on the basis of incorporation of amino-acid into polypeptide material. It seems reasonable, however, to predict that, under these conditions, mRNA is heterogeneous with respect to size (Okamuro & Busch[183]).

F I G U R E 10.

A drawing of a segment of tobacco mosaic virus.

(from Caspar, D.L.D. and Klug, A. (1962). Cold Spr. Harb. Symp. quant. Biol., 27, 1.

FIGURE 10



V. VIRUS STRUCTURE.

An outstanding characteristic of most viruses that are stable enough to survive purification is that, for a given virus disease, all the individual particles appear to have a well-defined and similar structure.

Viruses may infect bacteria, plant or animal cells, but a recent attempt at classification viruses as a whole (Lwoff et.al.[132]) has emphasised that structurally at least there is no clear division between viruses of one host type (e.g. bacteria) and another.

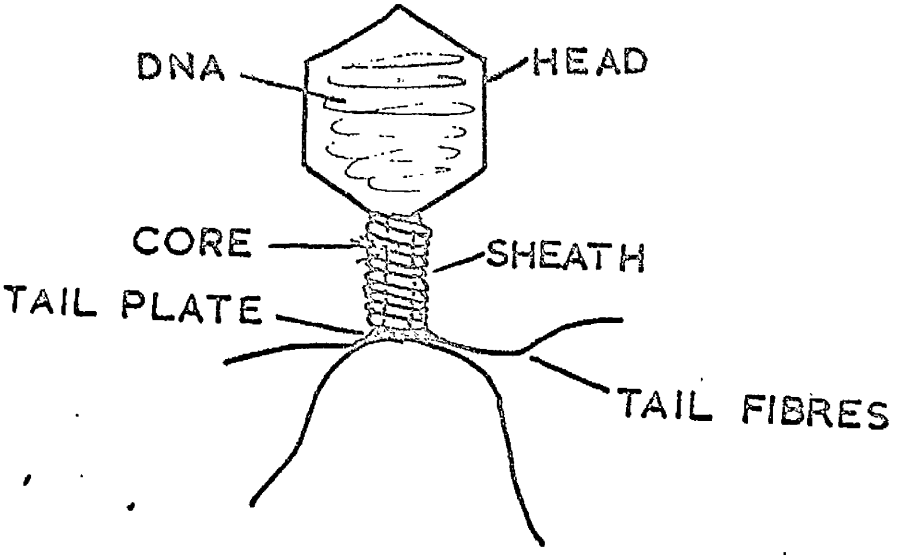
(a) Coat Morphology.

All known plant viruses contain RNA, but differ in the morphology of their protein coats. For example, T.M.V. has a helical capsid (Fig.10) 200 Å in diameter (Klug & Caspar[133]). The capsid is constructed from 2130 identical protein subunits, which envelope the nucleic acid completely and protect it. The RNA is single-stranded. The smaller TMYV, on the other hand, has a protein coat which exhibits cubic symmetry (Schmidt et.al.[134]) and which contains RNA packed in the hollow centre of the cube of protein (cf Fig.12a). The protein shell of TYMV contains subunits, the mechanism of packing of which in this and all the spherical viruses must conform to icosahedral symmetry (Caspar & Klug[135]).

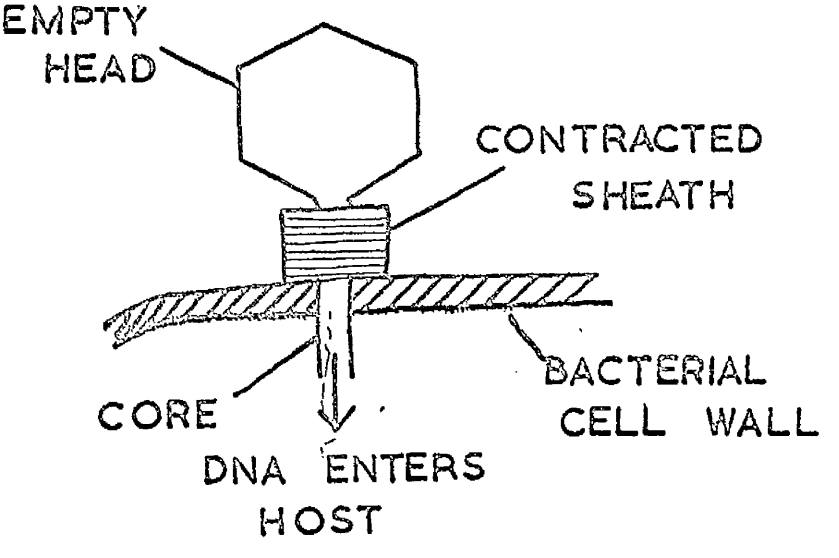
F I G U R E 11.

A representation of the structure of Bacteriophage T2.

FIGURE 11


DNA
HEAD
CORE
SHEATH
TAIL PLATE
TAIL FIBRES

(a)


EMPTY HEAD
CONTRACTED SHEATH
BACTERIAL CELL WALL
CORE
DNA ENTERS HOST

(b)

TYMV, thon, has a capsid made up from 32 capsomeres. These two viruses illustrate well the two types of virus capsid morphology generally found: a third, hybrid structure, is typified by bacteriophage (Fig. 11) (e.g. T-even coliphage.)

The bacterial viruses are structurally a diverse set: no helical bacteriophage particles have been isolated, but many of the smaller bacteriophages have cubic symmetry (Burdon [136]). Perhaps the typical bacteriophage structure however is that shown in (Fig. 11), a structure common to the T-bacteriophages, bacteriophages etc. (Williams & Fraser [137]) and constitutes a hybrid of helical and cubic symmetry. The nucleic acid is contained in the "head" portion of such structures. Bacteriophages have been isolated which contain single or double-stranded DNA or RNA

The RNA-containing animal viruses, the myxoviruses and the reoviruses, are structurally similar. The myxovirus group (e.g. influenza, Newcastle Disease Virus) exhibit helical symmetry, and contain single-stranded RNA, while the reoviruses are "spherical" and have double-stranded RNA as genome (Gomatos & Tamm [139]). RNA extracted from many of these viruses has been shown

F I G U R E 12.

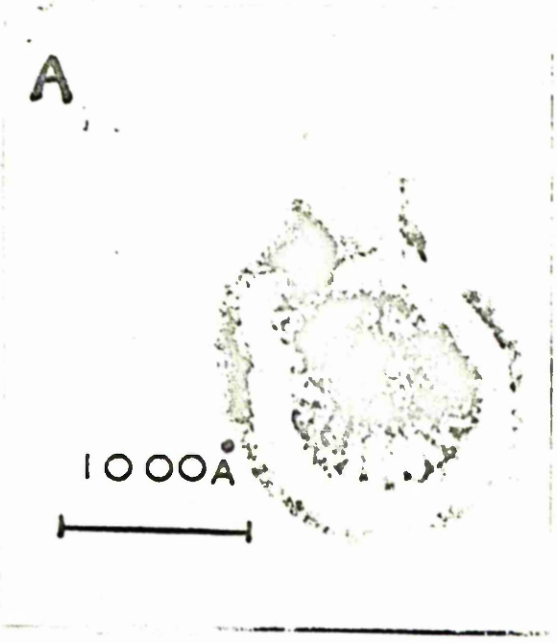
Morphological forms of Herpes Simplex virus

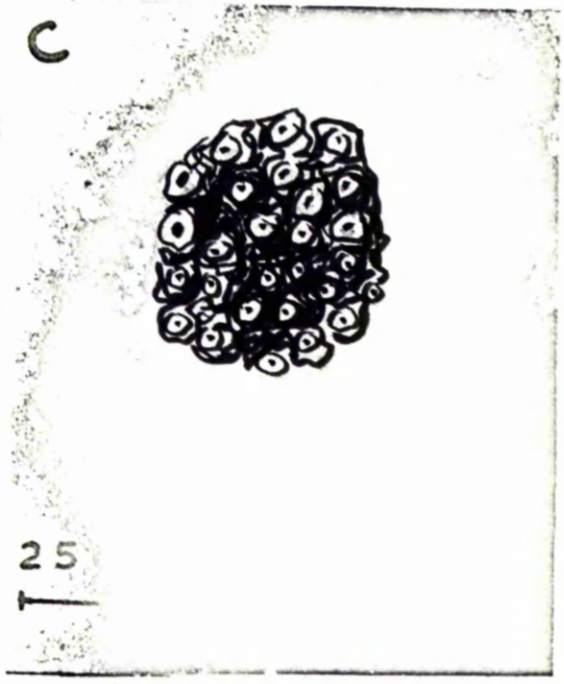
(a) "Full" enveloped particle.

(c) "Full" naked capsid.

(from Wildy,P. and Watson,D.H. (1962). Cold Spr. Harb. Symp. quant. Biol., 27,25.)

FIGURE 12





to be infective e.g. EMC (Huppert & Sanders [184]).

(b) DNA- containing Animal Viruses.

There are four main classes of DNA-containing animal virus, (Green [185]) poxviruses, herpesviruses, adenoviruses, and papovaviruses: all but the poxvirus group show cubic symmetry. Vaccinia virus, a large helical poxvirus, has a double-stranded genome of 80 x 10^6 M.W. units (Joklik [186]). It multiplies in the cytoplasm, unlike the tumour-producing viruses (polyoma, and papilloma) and unlike the adeno viruses which are spherical and contain double-stranded DNA of base composition similar to their host cell. A feature of the myxovirus, poxvirus and herpes virus groups is the high lipid content of the infective unit: this is due principally to the presence in these virions of a capsule of host-specific material e.g. (Nagington & Horne [187]). Recently Crawford [35a] has isolated a single-stranded DNA virus (MMV).

(c) Herpes Simplex Virus.

There are several viruses in the Herpesvirus group: all are medium-sized DNA-viruses showing several biological and morphological features. We will confine further definition to the Herpes simplex virus.

The Herpes simplex virion (Wildy et.al. [188]) consists of three main parts; (i) a core, containing the nucleic acid enclosed in (ii) a capsid (Fig.12c) surrounded by (iii) an envelope (Fig.12b). The core contains DNA (Epstein [189]) and no RNA (Russell [190]); the DNA has a high (G + C) content (68%) and behaves as double-stranded DNA in the presence of formaldehyde and on heating in solution (Russell & Crawford [191]). Estimation of the molecular weight of Herpes simplex virus DNA (Russell & Crawford [191]) by calculation from the sedimentation coefficient has given a value of 68×10^6 M.W.: this calculation may not be valid, however (Burgi & Hershey [192]). It has not proved possible to ascertain the number of DNA molecules per virion.

The icosahedral capsid contains 162 hollow elongated capsomeres. The number of protein subunits in each capsomere has not been determined nor has the possibility been investigated that the capsid contains other chemical components in addition to protein. It is known however, (Russell [190]) that herpes simplex virus contains 22% phospholipid and 1.6% carbohydrate, but it is believed that these compounds are derived from the envelope which surrounds the capsid. This

envelope is inconstantly present, variable in size and shape and periodically possesses projections (Wildy & Watson [193]). The envelope is derived from the host cell as shown by immunological techniques (Morgan et.al. [194]): conversely anti-herpes serum is active only on naked particles.

The separation of naked from enveloped forms of Herpes simplex virus by Smith (Smith [195]) has shown that only enveloped forms are infective, suggesting that the envelope is essential for infectivity: however (Wildy & Watson [193]) using a more indirect approach to the problem have evidence that the enveloped particle is merely a more efficient infective unit.

VI. BIOCHEMISTRY OF BACTERIOPHAGE T2 INFECTION.

In considering the infective process during bacteriophage infection we can recognise three events: attachment and penetration, replication and release.

(a) Phage Structure and Function.

Bacteriophage T2, which infects Escherichia coli, has a complex structure in common with all double-stranded DNA bacteriophages (Fig.11) (Williams & Fraser[137]) The polyhedral head, whose membrane is protein, and to which the tail is attached, contains the DNA of the phage. The tail comprises a core with a contractile protein sheath whose terminal is a hexagonal plate with long fibres, one fibre per face of the hexagon (Bronner et.al. [198]).

The first step in phage attachment is a random collision between the phage and its host: the site of attachment of phage is the cell wall (not the membrane (Zinder & Arndt [199]), and each phage has its specific receptor site (Fisher [200]). The tail fibres of the phage bind to the cell wall, and phage enzyme creates a gap in the rigid layer of the wall (Hayes [201]).

(b) Biochemical Events.

After attachment, the phage DNA is injected into

the bacterium while its protein coat remains outside. Hershey & Chase (Hershey & Chase[202]), in their famous blendor experiment first demonstrated this as follows: Phage T2 DNA was labelled with ^{32}P, and protein with ^{35}S Phage was allowed to absorb to host bacteria, and attached bacteriophage was removed by "shearing" in a blendor. They found that 80% of the ^{35}S was removed by this treatment, but only 20% of the ^{32}P. This has since been verified for other bacteriophages (Luria & Steiner[203]). The latent period lasts for about 25 minutes, and new intact bacteriophage particles are not formed until 12 - 15 minutes post infection (Doerman [204]). Bacteriophage T2 DNA replication starts after 6 minutes, and, after the DNA pool has reached a certain size, the DNA is irreversibly incorporated into bacteriophage heads (Hershey [205]); the replicative process does not extensively utilise the bacterial precursor pool but incorporates some material derived from the host DNA (Stent et.al.[206]).

Phage protein is synthesised exclusively from the medium (Kozloff et.al.[207]), and the first few minutes of infection are characterised (a) by the appearance of protein distinct from the bacteriphage coat-precursor protein (Manaloe et.al.[208]) and (b) by the abrupt cessation of bacterial protein synthesis

TABLE 1.

"Early Enzymes" formed in bacteriophage-infected Escherichia coli cells.

TABLE 1

"EARLY ENZYME"	Reference
deoxycytidine mono phosphate hydroxy methylase	211
deoxy hydroxymethyl cytidine 5'-phosphate kinase	211
DNA nucleotidyl transferase	214
glucosyl transferase	211
deoxycytidine pyrophosphatase	211
thymidylate synthetase	216
deoxycytidylate deaminase	215
deoxyribonuclease (activity increase)	212
thymidylate kinase (activity increase)	211

(e.g. β galactosidase (Benzer [209])). "Early" proteins have been found to include enzymes, some of whose activities are not found in uninfected cells e.g. deoxycytidylate, hydroxy-methylase (Flaks & Cohen[210]) and glucosyl transferases (Kornberg et.al.[211]) and others which represent a post-infective increase in activity e.g. DNase (Kunkee & Pardee [212]). This increase in activity nevertheless, represents the functioning of a new enzyme in infected cells.

A complete list of early enzymes is shown in Table 1.

(c) Macromolecular Synthesis.

Bacteriophage T2 contains no RNA, but RNA is synthesised in T2-infected Escherichia coli. This RNA fraction which has base ratios similar to bacteriophage DNA but not Escherichia coli DNA (Volkin & Astrachan[217]) and which forms a hybrid specifically with T2 DNA (Hall & Spiegelman [218]) has been termed messenger RNA.

(Brenner et.al.[219]) have shown that bacteriophage protein synthesis takes place on ribosomes which, before infection, had been the site of synthesis of bacterial protein.

When the DNA and protein pools constituting the various parts of the bacteriophage reach a certain

level, intact bacteriophage is assembled randomly from these components (Brenner [220]).

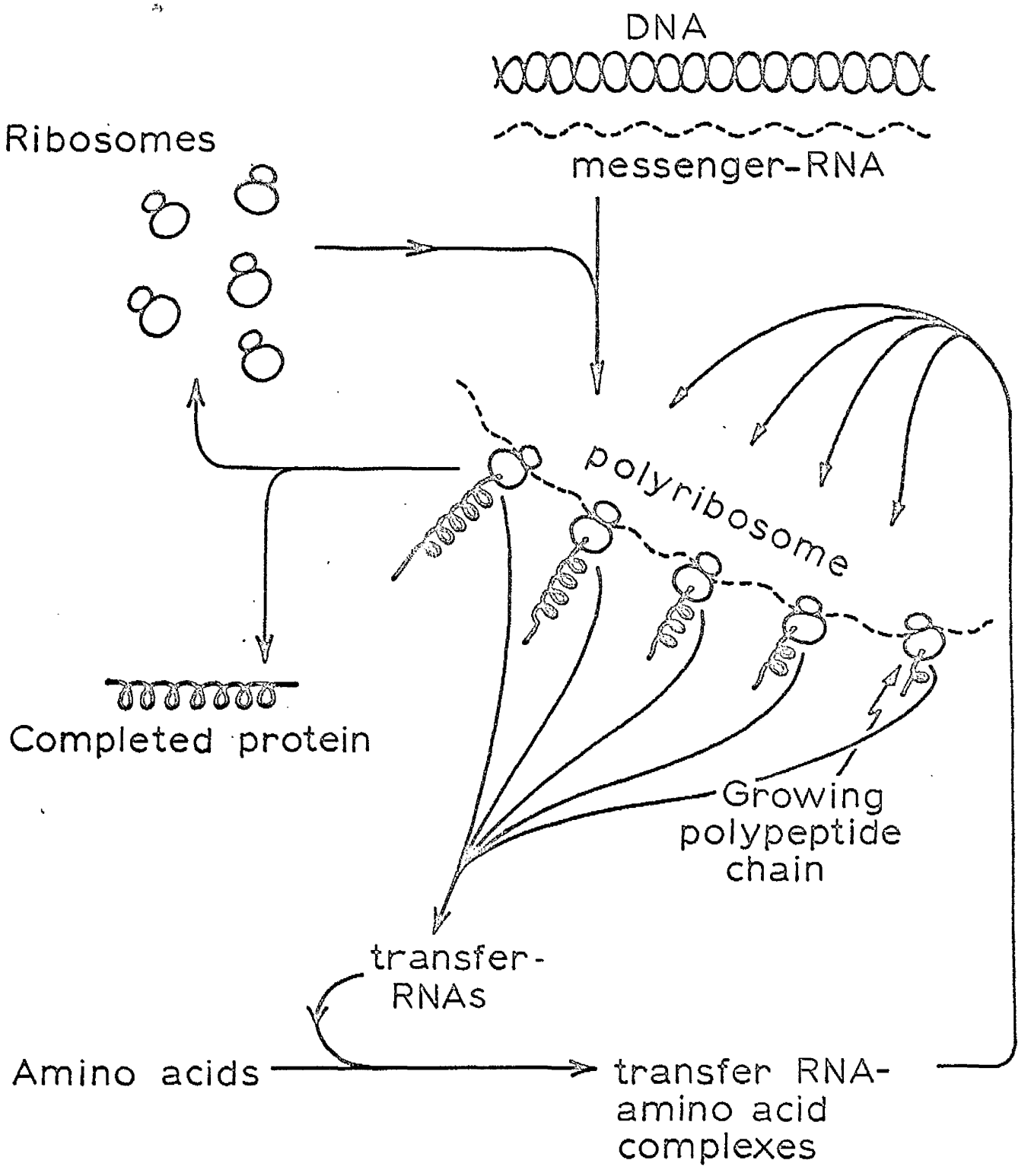
Under standard conditions, lysis of the host cell and release of mature virus takes place at a standard time after infection. (Streisinger et.al.[221]) have evidence that the process of lysis is at least initiated by the production of muramidase by the bacteriophage.

F I G U R E 13.

Nucleic Acid programming of protein synthesis in the normal cell.

(From Keir, H.M. (1965); Surgo, 22,3).

FIGURE 13


DNA
Ribosomes
messenger-RNA
polyribosome
Completed protein
Growing
polypeptide
chain
transfer-
RNAs
Amino acids
transfer RNA-
amino acid
complexes

VII. PROTEIN SYNTHESIS.

All species of RNA are transcribed from DNA, and all are necessary in the cell as components of the protein-synthesising system: this has been demonstrated in bacteria (Spiegelman [222]) and mammalian systems (Zamecnik [223]). Ribosomes - ribosomal RNA complexed with protein - have long been known to be the site of protein synthesis and (Nirenberg & Matthaei [224]) recognised that the information necessary for the synthesis of protein is not an integral part of the ribosome itself, but is in the form of an additional molecule, messenger RNA. It has been shown in work on the r_{II} locus of bacteriophage T4 that a unit of three nucleotides in the messenger RNA is probably responsible for the coding of a single amino acid (Crick et.al. [225]).

(a) Ribosomes.

(Roberts et.al. [226]) has demonstrated that in Escherichia coli, ribosomes are synthesised via two precursors: eosomes which contain little protein, and neosomes, with one half the protein content of mature ribosomes. On the basis of a theoretical argument Roberts (Roberts [227]) suggests that most cellular proteins

are synthesised on a template of ribosomal RNA (the cosome), and predicts that the remaining protein is translated from messenger RNA using a doublet code. However, the triplet code and messenger RNA mechanism of protein synthesis has received a great deal of support e.g. (Staehelin et.al.[228], Reichman [229], Soll et.al.[230]), and is widely accepted.

(b) Messenger RNA.

Messenger RNA is synthesised on a DNA template, with a base sequence complementary to only one of the DNA strands (Marmur et.al.[231]) it forms a loose complex with a variable number of ribosomes to form polyribosomes (Warner et.al.[232]) chiefly in the cytoplasm of the cell (Allfrey & Mirsky[233]). Polyribosomes have been demonstrated in a variety of organisms e.g. animal cells (Warner et.al.[232]) moulds (Phillips et.al.[234]) and bacteria (Staehelin et.al.[235]); they appear to be biologically ubiquitous.

(c) Polysomes.

The model of Rich (Rich et.al.[236]) has shown that the size of the polysome is related to the size of the messenger RNA and that each polysome contains only

one RNA strand: ribosomes attach themselves to polysomes and are released as protein synthesis takes place, there being a quantitative relationship between the number of nascent polypeptides and the size of the polysome. Polypeptide chains are probably released under the influence of GTP (Morris [244]).

Messenger RNA becomes associated with the 30S component of the ribosome, while molecules of amino acyl-tRNA associate with the 50S subunit. (Cannon et.al.[237]): two tRNA molecules may bind to the ribosome concurrently (Williamson & Schweet[238]).

(d) Transfer RNA.

Transfer RNA is bifunctional: each type accepts on the adenosine terminal a specific amino acid from a particular activating enzyme (Zamecnik[223]) and contains a base sequence such that it is able to recognise a triplet code word of the messenger RNA.

This enables the correct sequence of amino acids in protein to be attained.

There is at least one tRNA specific for each amino acid (Yamane et.al.[240]) and there is heterogenicity within an amino acid-specific population of tRNAs. In Escherichia coli e.g. five leucyl tRNAs

have been found and these have been shown not to participate identically in protein synthesis (Weisblum et.al. [242]): this is a direct consequence of code degeneracy (Crick [243]). Amino acyl tRNAs attach to ribosomes as dictated by messenger RNA, and transferase enzymes bring about peptide synthesis, releasing the tRNA (Nathans & Lipmann [245]).

(e) The Code.

The genetic codewords have now largely been assigned to amino acids, the existence of "nonsense codons" with chain terminating possibilities have been confirmed (Brenner et.al. [246]) but this will be discussed later. The code is non-overlapping and universal (Ochoa & Wettstein [247]) and there is evidence for the colinearity of gene and amino acid sequence (Yanofsky [248]).

Coding errors during protein synthesis may be minimised therefore by the requirement for correct recognition at three successive steps; i.e. DNA to messenger RNA, messenger RNA to tRNA and at the level of formation of amino acyl tRNAs.

Extensive degeneracy in the amino acid code may now be regarded as firmly established, and there

appear to emerge a few general patterns involving chiefly the third nucleotide (3') of a sequence: but degenerate initial nucleotides (5') have also been found (Nishimura et.al. [249]).

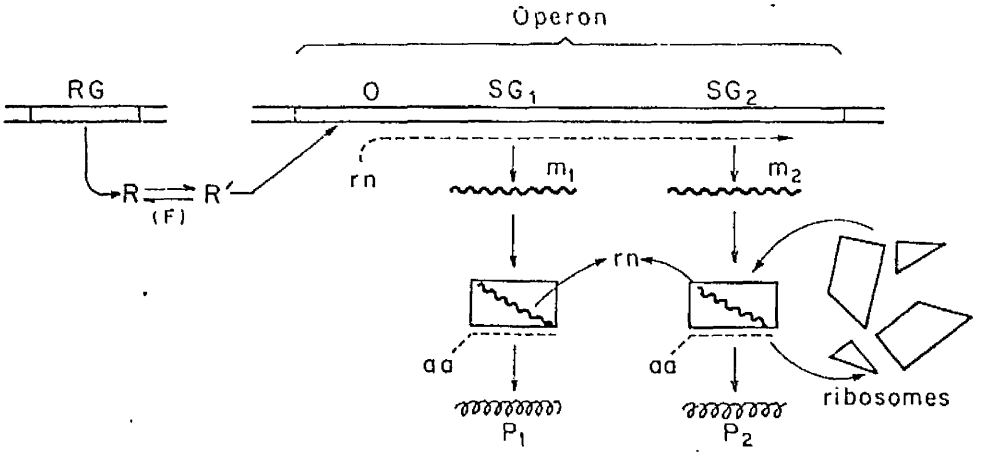
F I G U R E 14.

General model of the proposed regulation of enzyme synthesis.

RG: regulator gene; R: repressor converted to R' in the presence of effector F; O: operator; SG_1, SG_2: structural genes; rn: ribonucleotides; m_1, m_2: messengers made by SG_1 and SG_2; a.a.: amino acids; P_1, P_2: proteins made by ribosomes associated with m_1 and m_2.

(From Jacob, F. and Monod, J. (1961). Cold Spr. Harb. Symp. quant. Biol., 26, 193).

FIGURE 14



VIII. CONTROL OF MACROMOLECULAR SYNTHESIS.

(a) Bacterial Systems.

In bacteria, genes with regulating or control functions exist in two types - regulator genes outside the operon which govern the rate and conditions of synthesis of protein, and operator genes, within the operon, which control the rate of transcription of one or more structural genes adjacent to them in the chromosome: the operator gene is under the direct control of the regulator (Jacob & Monod [260]), Repression is a decrease while induction is an increase in the rate of protein synthesis (Vogel [261]): these regulatory phenomena are considered to depend on the product of the regulator gene and perhaps also on a substrate or product of the biochemical pathway controlled by the operon (Ames & Martin [262]) (Fig.14).

Experiments with cells heterozygous for a normal and a mutant regulator gene (Horuichi et.al. [263]) suggest that the regulator gene produces a specific soluble product, the repressor, which inhibits protein synthesis: the repressor may itself be a protein, or its production may involve a protein (Benzer & Champe [264]).

Lactose utilisation in Escherichia coli, for

example, requires a regulatory gene and three structural genes which are closely linked to each other genetically: these last correspond to β galactosidase, galactoside permease and thiogalactoside transacetylase (Jacob & Monod [265]); the regulator gene maps outside of the lactose operon (Pardee et.al. [266]).

However, it is still not clear whether the regulation of the operon occurs at the level of transcription or translation. (Hayashi et.al. [267]) in work on the lactose operon in Escherichia coli and (Attardi et.al. [268]) using several enzyme systems in the same bacterium showed an increase in messenger RNA corresponding to induced gene transcription after chemical induction; on the other hand, Yanagisawa has evidence that the reading of messenger RNA in Escherichia coli K12 Met⁻ takes place under the control of the inducer (Yanagisawa [269]) and suggests that the repressor may combine with the messenger RNA or a ribosome.

(b) Mammalian Cells.

Mammalian Cells.

In mammalian cells, however, the mechanism of regulation of protein synthesis is more obscure than is the case for the bacterial cell. Animal cell DNA, unlike bacterial DNA, is present in vivo as

nucleohistone and the presence of variable amounts of protein has been correlated with the degree of differentiation of the cell (Allfrey & Mirsky [270]).

(Allfrey & Mirsky (1962) [271]), using DNA-release criteria observed that 15 - 20% of the DNA of Calf thymus lymphocyte acted *in vivo* as primer for RNA synthesis. These results, and later work *in vitro* using isolated Calf thymus nuclei demonstrating the repression of DNA template function in transcription (Frenster *et.al.* [272]) led the authors to predict that histones may function as repressors on the DNA of higher organisms (Allfrey & Mirsky [270]). These polycationic histone proteins, bound to DNA, are postulated to interfere effectively with the action of RNA nucleotidyltransferase (E.C. 2.7.7.6.)

Recently, however, (Sonneborn & Zubay [273]) the effect *in vitro* of histone in depressing the activity of DNA dependent RNA nucleotidyltransferase has been re-examined, and the action of histone *in vitro* has been suggested to be due chiefly to an aggregation phenomenon rendering primer DNA insoluble. Furthermore, no release of histone from DNA can be observed during transcription in the chromosone puffs of *dipteran* larvae. (Swift[274]).

Even if we assume that histones act as repressors in higher organisms, the mechanism of derepression are as yet poorly understood. Frenster (Frenster [275]) has proposed a model based on the specific binding to and subsequent removal of a portion of histone from DNA by a specific derepresser RNA. Such derepression has been postulated as the mode of action of some hormones e.g. (Loeb & Wilson [276]).

Frenster's model of regulatory RNA may be relevant to a virus infected cell system, possibly in the opposite sense, and this will be discussed later in this thesis.

The Present Project.

Current knowledge, then, of the biochemical situations arising in a virus-infected cell is derived chiefly from extensive studies (Cohen; Kornberg; Spiegelman)[393] carried out in bacterial cells infected with T-even bacteriophage.

We may extrapolate these findings to the animal virus-infected system and conclude that when a virus particle successfully infects a host cell the viral genome first initiates synthesis of new nucleic acids, some of which then programme or induce qualitative or quantitative changes in the synthesis of proteins. The cell's metabolism will then undergo a sequence of often radical changes which eventually lead to the production of progeny virus particles. The host cell is normally destroyed by this process.

It is the aim of the present investigation to examine the synthesis of nucleic acids in BHK 21 (C13) cells (hamster kidney cells) infected by herpes simplex virus, and, if possible, to correlate this synthesis with other biochemical events concurrent in the system.

Herpes simplex virus is potentially a favourable virus to examine with respect to nucleic acid synthesis: its genome is relatively large, and, if entirely

functional, has the abilibty to code for many proteins.

SECTION II.

EXPERIMENTAL

MATERIALS.

Materials were obtained from the following sources:

RNase A was purchased from Worthington Biochemical Corporation, New Jersey, U.S.A.: Tris, DNase I and DNA (Salmon sperm) were purchased from Sigma Chemical Co., LONDON: RNA (Yeast), Bentonite, Analar grade Toluene, Analar grade Phenol and Analar grade Sodium dodecyl sulphate were purchased from BDH Ltd., POOLE, Dorset: CsCl (Analar) was purchased from Hopkins and Williams, Ltd., LONDON: PPO, POPOP, Napthalene, Dioxane (Scintillation grade) and Hyamine Hydroxide were purchased from Nuclear Enterprises, Ltd., EDINBURGH: ATP and CTP were purchased from Swarts Bioresearch Inc., N.Y., U.S.A: DEAE-cellulose powder and Whatman "3MM" paper were purchased from Whatman Ltd., LONDON: Cellulose acetate membrane filters, (0.45 μ pore size) were purchased from the Millipore Filter Corporation, BEDFORD, Mass., U.S.A.: Cellulose nitrate membrane filters, No. 50 were purchased from Membranfilter Gesellschaft, (G.m.b.H.) GOTTINGEN, Germany: Teflon-coated glass fibre paper was purchased from Joymar Scientific Inc., N.Y., U.S.A.: All radioisotopes were purchased from The Radiochemical Centre, AMERSHAM,

Bucks.: L-Amino Acids, (A grade) were purchased from Calbiochem. Inc., LOS ANGELES, U.S.A.: Kieselguhr (Hyflo Super Cel) was purchased from Koch Light Laboratories Ltd., COLNBROOK, Bucks.: Bovine Serum Albumin (Fraction V) was purchased from Armour Laboratories, EASTBOURNE: Agarose powder was purchased from L'Industrie Biologique Francaise, S.A., GENNEVILLIERS, France: Sephadex G.25 was purchased from Pharmacia Ltd., LONDON: Activated charcoal was purchased from Barnabey-Cheyney Inc., COLUMBUS, Ohio, U.S.A.: T1 RNase and Mitomycin C were purchased from SANKYO Chemical Co., TOKYO, Japan: the Peristaltic-type Microspump "T" was purchased from the Distillers Co., Ltd., (F.A.Hughes & Co., Ltd., EPSOM, Surrey.): All other chemicals (Analar grade) were purchased from BDH, Ltd., POOLE, Dorset or Hopkins and Williams, Ltd., LONDON.

1. VIRUS GROWTH, & HARVESTING CONDITIONS.

(a) Cell & Virus Growth.

In this study, two mammalian cell lines were used: (i) BHK21 (C13) (MacPherson & Stoker [303]) and (ii) HEp 2 (Moore et.al. [307]). The first of these, the hamster kidney cell, was used in all experiments with the exception of virus preparation. Virus stock was grown in HEp 2 cells (human epidermoid carcinoma) always.

The virus used was, except where detailed specifically, Herpes Simplex virus, strain α (a specially purified sub-strain of HFEM) (Vantsis & Wildy [308]). Cells were grown as monolayers in 80 ounce cylindrical bottles which rotated about their long axis in the presence of 200 ml. of medium. The bottles were gassed with 95% air and 5% CO_2. The medium routinely used was a modified Eagle's medium (8 parts) supplemented with 3% Tryptose phosphate (1 part) and unheated calf serum (1 part) (ETC) (Vantsis & Wildy [308]). Under these conditions the BHK21 (C13) cell population doubles every 12 hr.

1.

(b) Harvesting of cells & virus.

Cells were harvested in the following way: the medium was removed from the bottle and replaced with 25ml. of 0.2% Trypsin in 0.016MEDTA. After washing the cell sheet thoroughly with this, it was replaced with a fresh portion and the bottle incubated at 37° until the cell sheet was removed from the glass. The resulting cell suspension was poured into 2ml. of calf serum and washed with ETC. The final washed suspension, in ETC, was either (i) sub-cultured (ii) sub-cultured and infected <u>in situ</u> with Herpes Simplex virus at 1 to 10 plaque-forming units per cell (for virus stock growth only) or (iii) infected with Herpes Simplex virus at 10 to 20 plaque-forming units per cell during a shaking period of 20 to 30 min.; the cells with adsorbed virus were plated and grown as described above. After 40 to 48 hr. virus production and release had taken place and the progeny herpes particles were isolated as follows: the medium was removed from the bottle and the released virus it contained was sedimented at 19,000 rpm for 35 minutes in the No. 30 rotor of the Spinco Model L Ultracentrifuge.

The cell sheet was then removed with Trypsin and EDTA, sedimented by centrifuging at 500g. for 5 minutes, and exposed to ultrasonic vibrations in the presence

of 0.14M NaCl in 0.005M Tris HCl, pH 7.4.

1.

(c) Differential Centrifugation.

The sonicated material was centrifuged at 500g for 5 minutes to remove cell debris and the virus was sedimented in the Spinco Rotor No. 30 as above. This cycle of sonication and centrifugation was repeated three times and removed much cellular contamination.

1.

(d) Deoxyribonuclease and Ribonuclease Treatment.

The virus was then suspended in 0.05M Tris HCl pH 7.4 containing 10^{-3}M $MgCl_2$ and exposed to 50 µg./ml. each of DNase I and RNase for 30 minutes at 37°, simultaneously. The virus was sedimented as above, washed with the buffer, and stored at 4° in SSC.

2. PREPARATION AND USE OF FRACTIONATING COLUMNS.

(a) Brushite Column.

Brushite ($CaHPO_4.2H_2O$) was prepared according to the method of Tiselius et.al. (Tiselius et.al. [339]) and a column 10 to 20cm. by 2cm. diameter was packed and subsequently used under pressure from an air pump.

The eluting properties of the column were checked by passing through it a narrow band of bromocresol purple dye.

The column was washed with 50ml. of 0.1M K_2HPO_4/KH_2PO_4 buffer pH 7.0 and the viral suspension applied in a small volume (5 ml.) of the starting buffer. The column was further washed with this buffer and the virus was eluted by washing with 0.4M and then 1.0M K_2HPO_4/KH_2PO_4 buffer pH 7.0 at room temperature. The virus-containing fractions were easily identified visually by their opalescence.

2.

(b) Chromatography on Columns of MAK.

(i) Preparation of Methylated Albumin (MA).

Crystalline bovine serum albumin was N- methylated by incubation with methanol in an acid environment. Two methods were used (i) that of (Mandell & Hershey [59]) and (ii) the more refined technique of (Hayashi, Hayashi and Spiegelman [340]).

(ii) Formation of MAK.

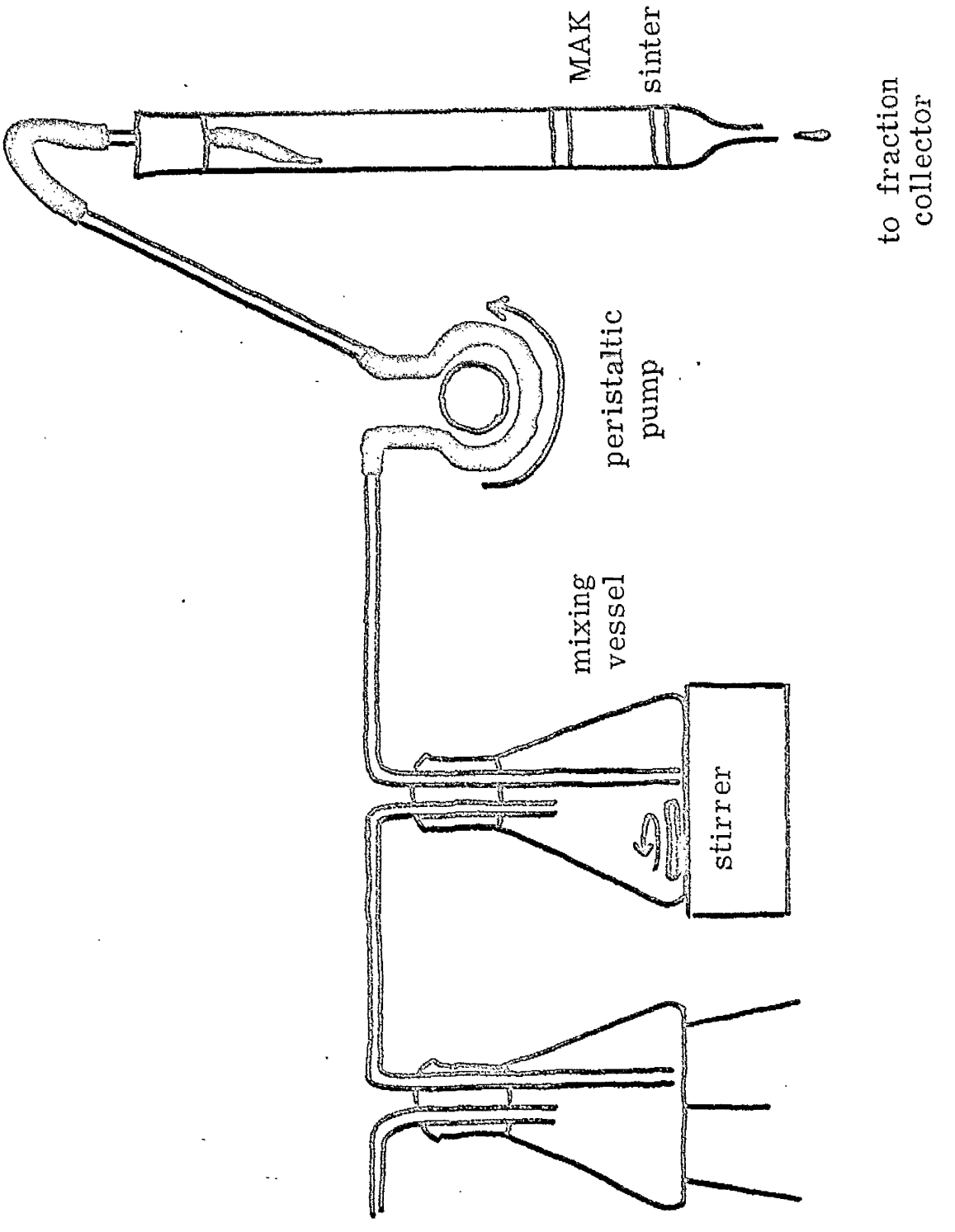
The method of (Mandell and Hershey [59]) was employed. A 1% aqueous solution of MA was stirred at 20° in the presence of boiled Kieselguhr (4g. per mg. MA)

F I G U R E 15.

Schematic diagram of the apparatus used in fractionation of nucleic acids on a Methylated Albumin Column.

The apparatus was used at room temperature, and the gradient-forming equipment shown gave an essentially linear gradient for elution.

FIGURE 15


MAK
sinter
to fraction collector
peristaltic pump
mixing vessel
stirrer

in 0.1M NaCl. The resulting MAK was stored at 4^{o} for a maximum period of 2 weeks.

(iii) The MAK Column.

The column of MAK was set up in a glass tube of uniform bore fitted with a sintered glass plate. MAK was packed under pressure in the tube using an air-pump, and a suspension of Kieselguhr ($\frac{1}{3}$ volume of the MAK used) similarly packed on top of this.

The column was washed with ten column volumes of 0.1 to 0.2M NaCl in 0.05M NaH_2PO_4/Na_2HPO_4 buffer pH 6.3 under pressure from a peristaltic pump. The flow rate was maintained at 2 - 3 ml./minute.

DNA or RNA was applied to the column at 20 µg./ml. in the above buffer and at a flow rate of 1 ml./minute. The adsorbed nucleic acid was eluted at room temperature with a linear gradient of NaCl in 0.05M sodium phosphate buffer pH 6.3, normally from 0.15M to 1.2M for DNA or whole cell RNA and from 0.15M to 0.80M for tRNA. The apparatus is shown in Fig. 15, and the flow rate of the column was maintained at 1 - 2 ml./minute using the peristaltic pump.

Alternatively, elution may be achieved by washing the column with batches of successively greater NaCl

concentration. This is a less satisfactory method.

Fractions were estimated directly for absorbancy at 260 mu and the gradient of NaCl calculated using the Abbe refractometer (Methods Sect. 6(e)).

2.

(c) Fractionation on "Sephadex" G25 Columns.

The dry gel was mixed with 0.01M Tris-HCl buffer pH 7.5 (1 g./100 ml.) and allowed to swell overnight at 4°. The smaller gel particles ("fines") which had not sedimented were removed by decantation, the remainder was stirred in fresh buffer and the removal of "fines" repeated.

The gel, as a slurry, was packed by gravity into a column equipped with a sintered glass disk at its base. Material to be fractionated was applied in small volume and eluted with 0.05M NaCl in the above Tris buffer under gravity flow at 4°.

2.

(d) Columns of DEAE-Cellulose.

DEAE-cellulose powder was suspended in 0.01M Tris-HCL buffer pH8 (5g./100 ml.) and "fines" were removed as above. The cellulose was washed successively in a

Buchner funnel with 0.1N HCL, distilled water, 0.1N NaOH and finally equilibriated with the above buffer. It was stored at 4^{o} in 2M NaCl, as the particle size becomes smaller with increasing ionic strength.

For use, the slurry in 2M NaCl was packed under air pressure into a column with a sintered glass outlet and washed under pressure with 0.02M Tris-HCl pH 7.5, to remove the NaCl.

Elution of material from the column-protein, sRNA or oligonucleotides - was carried out under gravity flow using either batches or a gradient of increasing salt concentration. As a rule, fractionation was carried out at 4^{o}.

3. ISOLATION OF DNA.

(a) Method of Marmur.

Marmur's (Marmur [284]) method, developed for the isolation of bacterial DNA, consists of three treatments (i) the suspension of DNA-containing material in SSC (cells, virus, etc.) is made 2% with respect to SDS and 1M with respect to $NaClO_4$ to dissociate DNA from protein (ii) it is then shaken exhaustively with an equal volume of $CHCl_3$/iso-amyl alcohol (50:1 v/v) to remove the protein by precipitation (iii) the deproteinised solution, containing DNA, has 2 volumes of ethanol carefully

layered on its surface: the skeins of DNA formed at the interface are "spooled" onto a fine glass rod and dissolved in 1/10 SSC. This DNA may be freed of RNA by incubation with RNase and deproteinisation, or by preferential precipitation of the DNA in iso-propanol. All steps are carried out at room temperature.

3.

(b) The Method of Russell [341]

This method is similar to the above procedure except that (i) the lysate in $NaClO_4$ is 1% with respect to SDS and is shaken for 5 minutes before addition of $CHCl_3$/iso-amyl alcohol and (ii) the entire procedure is carried out at 4^o.

3.

(c) A Phenol Method (Siato & Miura [342]).

In this procedure, the DNA-containing material in SSC is made 2% w.r.t. SDS and shaken in the presence of water-saturated, freshly distilled phenol (pH9) until protein-free. The DNA is "spooled" as above and treated with 50 µg./ml. RNase for 30 minutes at 37^o. Deproteinisation with phenol is again employed, and the final DNA solution is freed from residual phenol by

extracting it with ether and removing this by gassing with N_2.

3.

(d) "Modified Marmur" Procedure.

The starting material, 2% in SDS, 1M in $NaClO_4$ and in SSC was gently shaken at 37° for 1 - 2 minutes. It was then twice deproteinised with $CHCl_3$/iso amyl alcohol and the DNA "spooled" from 67% EtOH. If small amounts of DNA were present, centrifugation was employed in place of "spooling". The DNA was dissolved in SSC and treated with DNase-free RNase at 20 µg./ml. for 30 minutes at 37°. Protein was removed by shaking this solution with an equal volume of water-saturated, freshly distilled phenol containing 0.1% 8 - OH quinoline.

Further exhaustive deproteinisation was carried out using $CHCl_3$/iso-amyl alcohol; this also served to remove the residual phenol. The DNA was finally "spooled" or precipitated from 67% ehanol and stored in SSC at 4° with a few drops of $CHCl_3$.

4. PREPARATION OF BENTONITE.

Bentonite powder was purified by the method of (Singer and Frankael-Conrat [363]) as follows: the powder

was suspended in 0.1M EDTA (5g. per 100ml.) for 48 hours at room temperature, and centrifuged at 45,000g. for 10 minutes. The top half of the pellet was removed and suspended in 0.01M sodium acetate buffer pH5.2 and stored at 4^{o} after sterilisation.

5. PREPARATION OF RNA.

(a) The method of Kirby (343)

The cells were suspended in 8 volumes of 0.015M naphthalene-1.5-disulphonate (pH 6.8) and shaken with an equal volume of 90% phenol containing 0.1% 8-OH quinoline: this was repeated until the aqueous phase was protein-free. The RNA was finally precipitated from 67% EtOH, dialysed and stored at -70^{o}.

(b) The method of Eason, Cline and Smellie (343)

The RNA-containing material was made 0.1% with respect to SDS and 0.2% with respect to bentonite in 0.01M sodium acetate buffer pH 5.2 and deproteinised exhaustively with 90% phenol. The RNA was precipitated from 2% sodium acetate and 67% EtOH, taken up in 0.01M Tris-HCl buffer pH $7.5/10^{-3}$M $MgCl_2$ and treated with ether to remove phenol. DNase was added to 15 µg./ml. and incubation was carried out for 30 minutes at 37^{o}.

RNA was finally precipitated as above and dialysed against 0.05M NaCl/0.01M KCl/10^{-3}M $MgCl_2$ in 0.01 sodium acetate buffer pH 5.2. The modifications incorporated for use in the present work were:

(i) the phenol used was freshly-distilled

(ii) residual phenol was not removed before DNase treatment and

(iii) after DNase treatment the preparation was exhaustively deproteinised with phenol, which was removed in the subsequent dialysis.

5.

(c) Preparation of sRNA.

The washed cell preparation was suspended in 5 volumes 0.35M sucrose/0.025M KCl/5 x 10^{-3}M $MgCl_2$ in 0.05 Tris-HCL buffer pH 7.5 and disrupted by ultrasonic vibrations. Cellular particles were removed at 105,000g and the supernatant taken to pH 5.2 with 0.5M acetic acid at 4^{o}. The precipitate, obtained by centrifugation at 1500g for 10 minutes, was dissolved in 0.05M Tris-HCl buffer pH 7.2 and deproteinised with phenol (see section 5a). The sRNA was then precipitated from 2% sodium acetate and 67% ethanol. It was finally dialysed against 14 l. of water at 4^{o} for 12 hr. and stored at -70^{o}.

F I G U R E 16.

Calibration curve for Protein Estimation. The protein used was bovine serum albumin and the estimation method was that of Lowry et.al.[345].

F I G U R E 17.

Calibration curve for DNA estimation. Calf Thymus DNA was used and the method of Gorlotti was employed.[354]

FIGURE 16

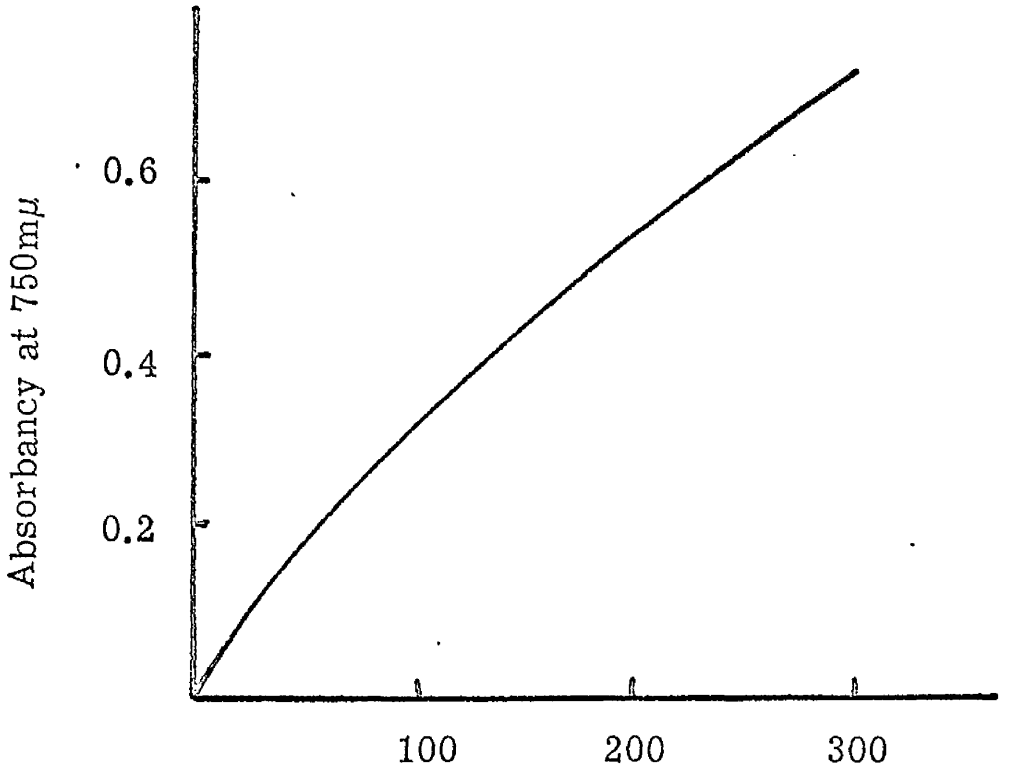
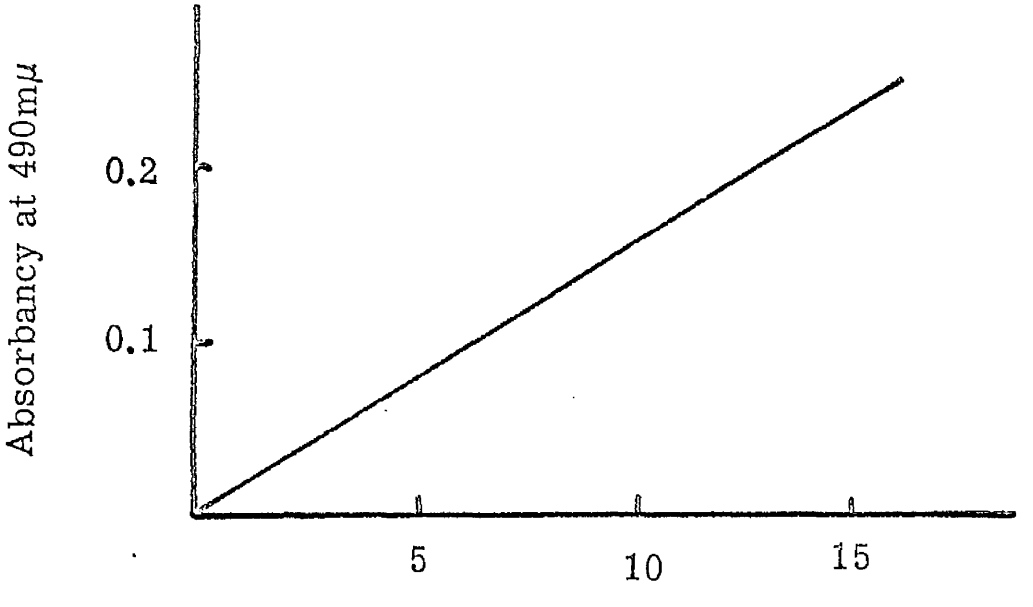


FIGURE 17



This is essentially the method of (Hoagland et.al. [344]), and was used as described except that bentonite was added to 0.2% in the initial lysate.

5.

(d) Further purification of sRNA.

sRNA prepared as above was further purified on columns of DEAE-cellulose or MAK (Methods Sects. 2(d), 2(b)(iii)). The appropriate fractions were pooled and the salt removed by dialysis: the salt - free solutions were concentrated by lyophilization and the sRNA was precipitated with sodium acetate and ethanol in the standard way.

The final preparation was dissolved in and dialysed against water and stored at -70°.

6. QUANTITATIVE ESTIMATION PROCEDURES.

(a) Estimation of Protein (Lowry et.al. [345]).

The protein solution (5 - 500 µg./ml.) was incubated at room temperature in the presence of $CuSO_4$, NaK tartrate, Na_2CO_3 and NaOH. Folin-ciocalteau reagent was added, and, after a further incubation, the absorbancy of the resulting blue solution was measured at 500 mµ or 750 mµ.

The method was calibrated using crystalline bovine

serum albumin and the curve is shown in Fig. 16.

6.
(b) Estimation of DNA (Ceriotti[354]).

The DNA solution (5 - 25 µg./ml.) was boiled in the presence of indole and HCL and the non-specific colour formed was removed with $CHCl_3$. Sample absorbancies were read at 490 mµ. A DNA standard solution was made up using a 1mg/ml. solution of calf thymus DNA in 0.1N NaOH. One ml. of this was diluted with 0.5N perchloric acid, heated at 70° for 20 minutes, and diluted to 50ml. with the same acid. The total phosphorus content of the solution was measured using the Allen phosphorus estimation procedure (Allen[355]).

The Ceriotti calibration curve is shown in Fig. 17.

6.
(c) The Orcinol Method of Estimation of RNA.
The Orcinol[368] Method of Estimation of RNA.
(Schneider[368]).

The test solution (25 to 200 µg./ml.), was incubated at 100°with the orcinol/$FeCl_3$ reagent, and the resulting colour estimated at 660 mµ.

The calibration curve (not shown) was constructed using commercial yeast RNA.

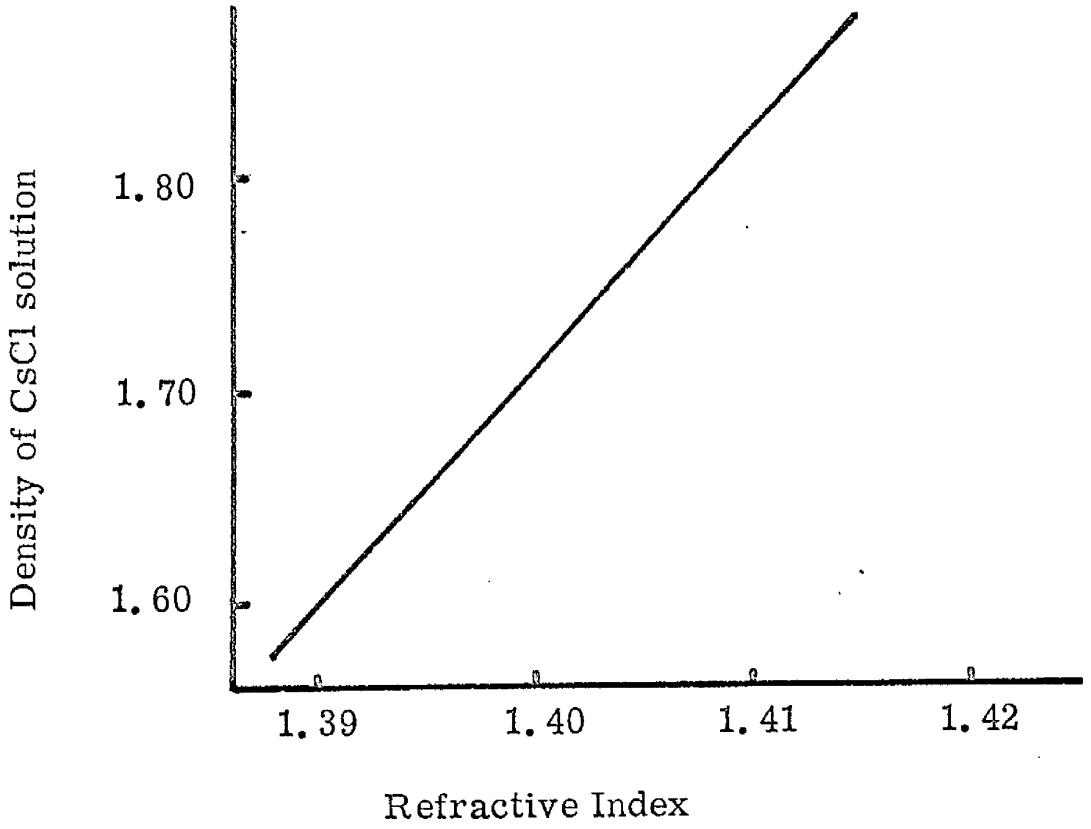
6.
(d) Estimation of DNA and RNA using a modification of the Schmidt - Thannhauser technique.

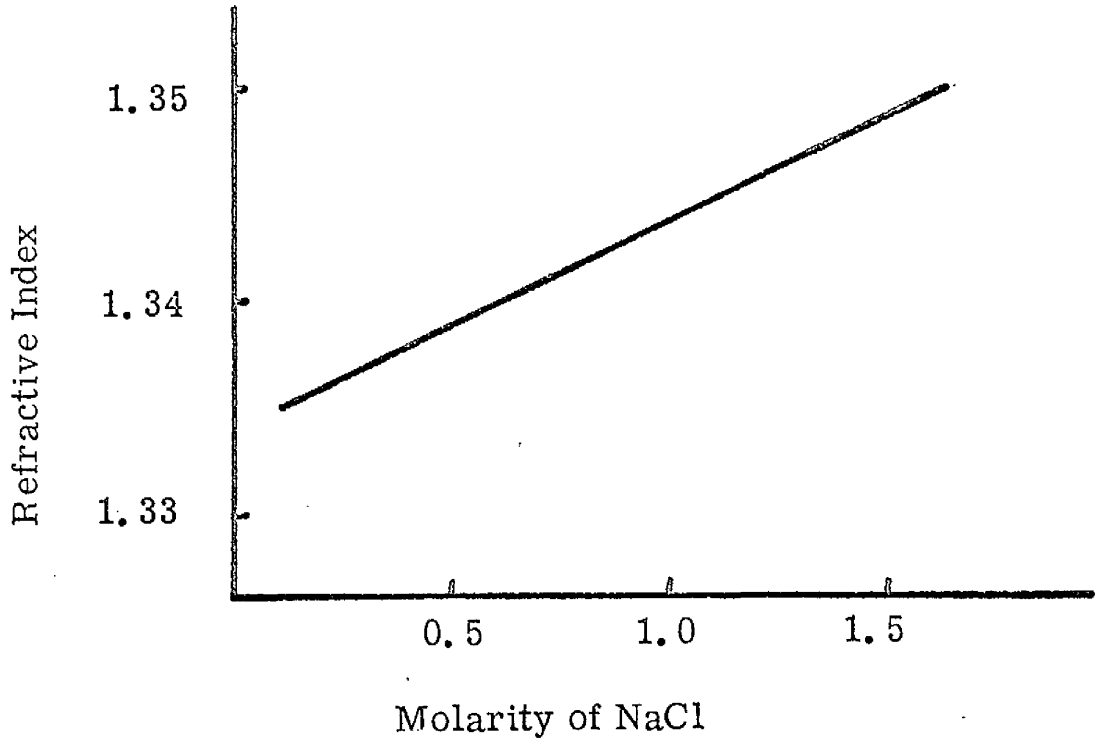
EXERCISE 30.

The refractive indices of solutions of NaCl and CuSO$_4$ as functions of concentration.

The refractive index of each was measured in the Abbé refractometer.

FIGURE 18


Density of CsCl solution
1.80
1.70
1.60
1.39
1.40
1.41
1.42
Refractive Index


Refractive Index
1.35
1.34
1.33
0.5
1.0
1.5
Molarity of NaCl

(Flock & Munro [350]).

A 1 in 20 (w/v) homogenate of cells was precipitated with cold perchloric acid. The RNA was then removed from the precipitate as soluble oligonucleotides after digestion with KOH and was estimated by its absorbancy at 260 mμ. The remaining DNA was dissolved in KOH and assayed using the Ceriotti procedure (Methods Sect. 6(b)).

6.
(e) Estimation of NaCl and CsCl.

Salt solutions possess a refractive index higher than that of water, and a graphic relationship between salt concentration and refractive index can be obtained. Curves for NaCl and CsCl are shown in Fig. 18. 0.1 ml. portions of salt solution were examined using the Abbe refractometer.

6.
(f) Cell Fractionation Method.

Cells were fractionated into nuclear and cytoplasmic preparations by the method of (Becker and Joklik [100]). The cells were suspended in hyoptonic medium, and, after 10 minutes, were disrupted in a Potter homogeniser. The homogenate was centrifuged at 600g for 2 minutes. The supernatant was termed

the cytoplasmic fraction; the pellet, after two washes in the same medium, yielded the nuclear fraction. The method was modified by the inclusion of 8-OH quinoline, (an RNase inhibitor) at saturation in the suspending medium prior to isolation of RNA.

6.(g) Ribonuclease Assay.

The system of (Brody [356]) involves incubation of the RNase fraction with RNA (free of acid-soluble material) at pH 7.5 and 37°. The reaction mixture is then treated with trichloracetic acid and lanthanum acetate, and the amount of RNA rendered acid-soluble is estimated by measurement of absorbancy at 260 mμ. This method was modified by the use of perchloric acid in place of trichloracetic acid, which absorbs at 260 mμ, and the use of bovine serum albumin as co-precipitant for the RNA.

6.(h) Liquid Scintillation Counting.

Material labelled with ^{3}H or ^{14}C was routinely assayed for radioactivity in the Nuclear Chicago Model 725 or Packard series 4000 liquid scintillation spectrometer, using the following scintillation fluids:

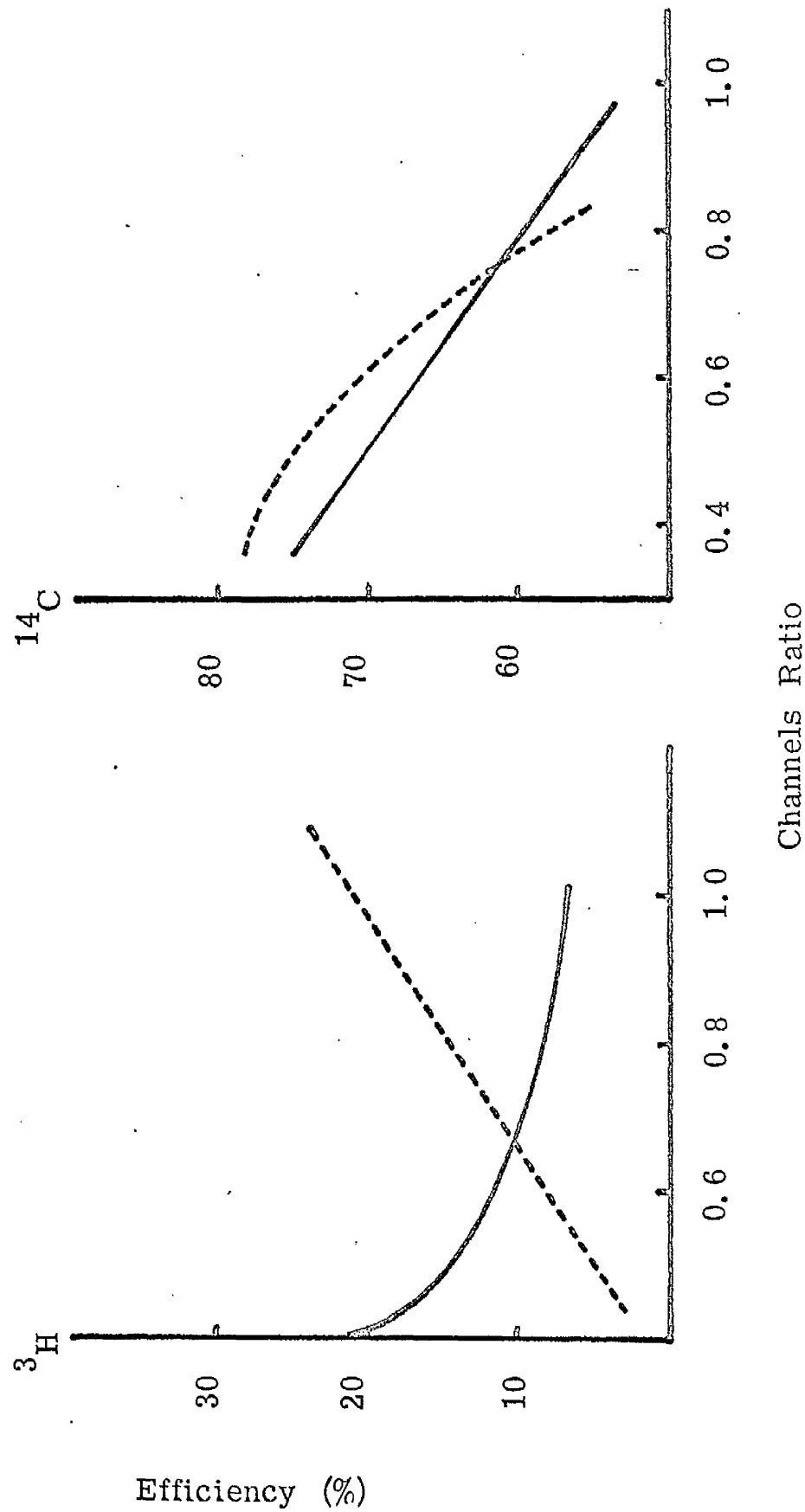
F I G U R E 12.

The counting efficiencies of ^{14}C and ^{3}H in the liquid scintillation spectrometer as a function of the channels ratio.

_____ Packard series 4000 counter

- - - - - Nuclear Chicago Model 725 counter

FIGURE 19


^{14}C
80
70
60
0.4
0.6
0.8
1.0
^{3}H
30
20
10
0.6
0.8
1.0
Efficiency (%)
Channels Ratio

(i) Dioxane-based scintillator was used for samples in aqueous media. It consists of 10% naphthalene, 0.7% PPO and 0.03% POPOP in scintillation grade dioxane. It has the disadvantages of being severely quenched by O_2 and of being useful only when the sample salt concentration is very low.

(ii) Toluene-based scintillator, 0.5% PPO and 0.03% POPOP in Analar toluene was used in conjunction with material precipitated onto filter desks.

Using these fluids, the efficiency of counting was estimated by the channels ratio method. Calibration curves are shown for ^{3}H and ^{14}C in Fig. 19. The curves are identical for either scintillation fluid.

(iii) Preparation of samples for counting. Samples were assayed for radioactivity using one of three methods (a) adding aqueous samples directly to dioxane-based scintillator, (b) precipitating the material with trichloracetic acid in the presence of a previously determined optimal amount of DNA on cellulose nitrate or cellulose acetate membrane filters, drying those at 50^{o} for 120 minutes and immersing them in 10 ml. of toluene-based scintillator, or (c) precipitating the sample as above but on a disk of teflon-coated glass fibre paper which was then dried with ethanol.

The dry disk was incubated with 0.05M Hyamine hydroxide in 10 ml. of Toluene-based scintillator for 10 minutes at 57°. This treatment solubilised the precipitated material, and was necessary because the teflon-glass fibre disk is opaque in toluene. (Burdon [357]).

Using these methods, efficiencies of counting for ^{3}H were 20 - 25% and for ^{14}C were 60 - 70% routinely.

7. FRACTIONATION OF RNA.

(a) Treatment of Sucrose with Bentonite.

Sucrose solutions to be used in the fractionation of RNA were routinely treated with bentonite to remove possible RNase contamination (Sanders [349]).

A 25% (w/v) sucrose solution was shaken with 0.01% bentonite at room temperature for 10 minutes. The bentonite was removed by centrifugation at 44,000g for 20 minutes. The sucrose solution was stored at -20°.

7. (b) Sucrose Gradient Analysis.

Both the sample and the sucrose were dissolved in 0.01M sodium acetate buffer pH 5.2 containing 0.05M NaCl, 0.01MKCl and 10^{-3}M $MgCl_2$. The sucrose gradient (5 - 20% or 5 - 25% w/v) was prepared using the device

F I G U R E 20.

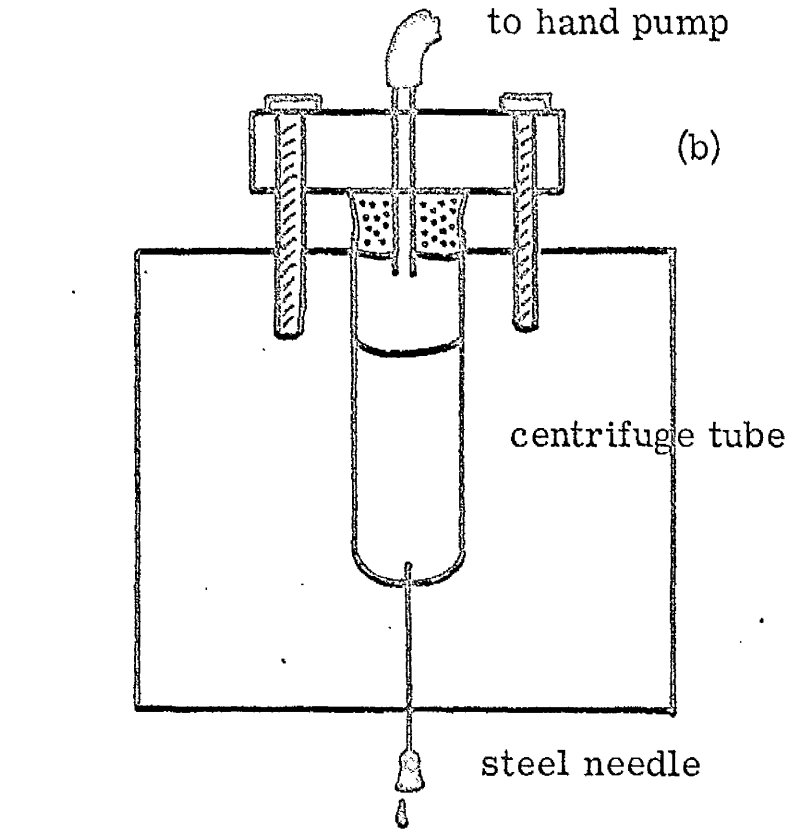
(a) Device for constructing a linear gradient of solute, generally sucrose.

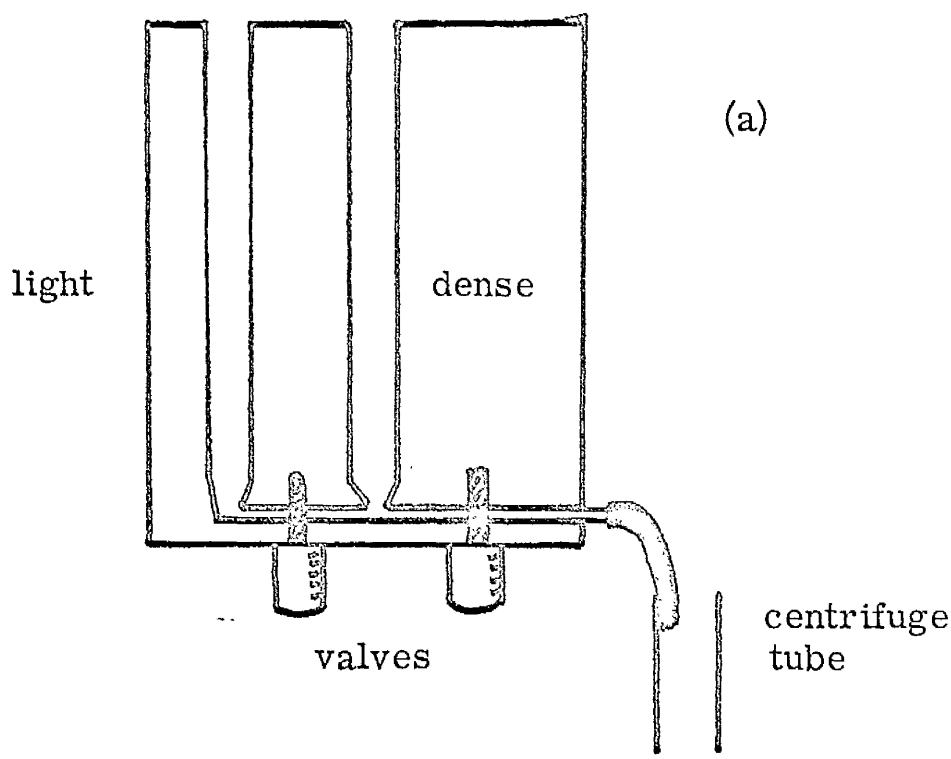
The "dense" column, filled with 20 or 25% sucrose, is continuously mixed by an air-stream: the "light" column contains 5% sucrose. The tube is filled over a period of 10 minutes at 4°.

(b) Harvesting apparatus for gradient analysis.

The tube is held in place by a clamp, and the rate of elution is kept constant using the hand-pump.

FIGURE 20


to hand pump
(b)
centrifuge tube
steel needle


(a)
light
dense
valves
centrifuge tube

drawn in Fig. 20a. Material to be fractionated was applied with a micropipette in small volume to the top of the gradient. The gradient was centrifuged at 21,000 r.p.m. for 12 hours or at 39,000 r.p.m. for 3 hours in the SW39 rotor of the Spinco Model L ultracentrifuge. Fractions of 0.1 ml. (2 drops) were collected by puncturing the base of the tube with a steel needle using the apparatus in Fig. 20b. Each fraction was usually diluted to 0.7 ml. with distilled water and estimated for absorbancy at 260 mu and for radioactivity.

All operations were carried out at 4°C.

7.
(c) Agarose Gel Electrophoresis (McIndoe [348]).

A 2% (w/v) slurry of Agarose in 0.02M Tris/0.005M Citric Acid, pH 7.9 was heated to 80° to dissolve the gel. After cooling to 40°, the gel was poured into a perspex tray (14cm. x 8cm. x 0.5cm) equipped with a mould which gave 1mm. x 10mm. sample wells. The electrode chambers contained 0.2M Tris-citrate buffer pH 7.9. A 50 µg. sample of whole cell RNA was applied to the wells and the gel subjected to a potential drop of 10v./cm. for 90 minutes. The RNA in the gel was fixed and stained in 2% aqueous Toluidine blue. The

excess stain was removed in water. When sRNA alone was to be fractionated, 4% gel (w/v) was used.

7.(d) Recovery of RNA from a Sucrose Gradient.

RNA from a sucrose gradient analysis (Methods Sect. 7(b)) was recovered by pooling the appropriate fractions and precipitating the RNA with sodium acetate (2%) and ethanol (67%). However, this method applied only if the RNA content of the fractions was high. If this was not the case, the pooled material was dialysed exhaustively against distilled water and dried from the frozen state. The residue was dissolved in a small volume of buffer solution and dialysed as above.

7.(e) Hydrolysis of RNA (Decker & Joklik [100]).

RNA was hydrolysed to its constituent nucleoside monophosphates by incubation at 37° for 18 hours in 0.3N KOH. The pH was adjusted to a suitable level (e.g. 7.0) by addition of perchloric acid to the hydrolysate at 0°. The precipitated $KClO_4$ was removed by centrifugation.

7.(f) Electrophoresis of RNA Nucleotides.

(Bell [391]).

The nucleoside monophosphates derived from RNA as above were adjusted to pH 3.5 with perchloric acid and applied in 100 µl. portions to one end of a 60 cm. x 15 cm. strip of Whatman 3MM paper. The paper was electrophoresed for 50 minutes at 3.500V (60V./cm.) in 0.07M ammonium formate buffer pH 3.3, dried, and the nucleotide spots marked under ultraviolet light. The spots were cut out, the material eluted from them with 0.1M HCL and evaporated to dryness on stainless steel planchettes. These were assayed for radioactivity (^{32}P) in the Nuclear Chicago gas flow counter.

8. FRACTIONATION OF DNA.

(a) Purification of CsCl.

Commercial CsCl (Analar) is contaminated with material which absorbs at 260 mµ. This was removed in the following way. A saturated solution of CsCl (50 ml.) was passed slowly through a column (10 cm. x 2 cm.) of activated charcoal which had been washed in distilled water. The eluate, which had an absorbancy at 260 mu of 0.01 - 0.02 and was evaporated to dryness in a vacuum desiccator and stored in an air-tight jar. The column process was repeated several times if

necessary.

8.
(b) CsCl Density Gradient Analysis.

The DNA sample was made 1.70 gm./ml. by adding purified, solid CsCl. The density was checked using the Abbe refractometer (Methods Sect. 6(e)) and centrifugation in the SW39 rotor at 33,000 r.p.m. for 3 days (Model L ultracentrifuge) or in the analytical rotor at 59,780 r.p.m. for 18 hours (Model E ultracentrifuge) was carried out at 25°C.

After equilibrium had been attained, the gradient was harvested (Model L) and assayed using technique detailed in Section 7(b). The Model E analysis was photographed at equilibrium using ultraviolet optics.

9. HYBRIDISATION OF DNA WITH RNA.

(a) Rapidly-Labelled RNA.

This technique was based on the method of (Hall and Spiegelman [172]). Purified rapidly-labelled RNA of high specific activity was mixed with heat-denatured DNA (normally 60 µg.) in SSC at 73°. After incubation at this temperature for 10 minutes, the mixture was held at 65° for 2 hours and then cooled slowly to 25° over a period of 12 - 15 hours. The

formed hybrid was stable at this temperature and was fractionated using CsCl density gradient centrifugation (Methods Sect. 8(b)).

9.
(b) Soluble RNA.

The method used was that of (Giacomoni and Spiegelman [353]). The DNA:RNA mixture was incubated for 2 hours in the presence of Mg^{2+}, and subsequently cooled rapidly in ice. This hybrid may be fractionated as above. (Section 8(b)).

10. PREPARATION OF AMINO-ACYL-tRNA SYNTHETASE.

(a) Enzyme Purified on DEAE-Cellulose.

This enzyme was prepared from cells by the procedure of (Ishida and Miura [358]). Cells were disrupted by ultrasonic vibrations in 0.25M sucrose containing 0.05M KCl and 5×10^{-3}M Mg^{2+}. and centrifuged at 105,000g for 2 hours. The top two-thirds of the supernatant fluid were removed and applied to a DEAE-cellulose column (15cm.x 2cm.) equilibriated with 0.02M Tris-HCL buffer pH 7.5 - 0.01M 2-mercaptoethanol. After thorough washing of the column with this buffer, the enzyme was eluted in 0.3M KCl containing the same concentrations of tris-HCl and mercaptoethanol and was

not normally stored. This procedure removed sRNA from the enzyme preparation. All operations were carried out at 4°.

10.
(b) Crude Amino Acyl-tRNA Synthetase.

Cells were broken ultrasonically in the presence of 0.02M Tris-HCl buffer pH 7.2 containing 0.015M KCl, 0.01M 2-mercaptoethanol and 0.005M $MgCl_2$ and centrifuged at 105,000g for 2 hours. The supernatant fluid was dialysed for 1 - 2 hours against two changes of 3l. of the above buffer, to remove free amino acid, and was used immediately.

11. PREPARATION OF AMINO ACYL tRNA.

(a) Removal of Amino Acid from Aminoacyl tRNA (Sarin and Zamecnik [359]).

The sRNA preparation was dissolved in 1.8M Tris-HCl buffer pH 8 and incubated at 37° for 30 minutes. The sRNA was then precipitated using 80% ethanol and dialysed exhaustively against distilled water.

11.
(b) Synthesis of Aminoacyl tRNA (Muto, Miura, Hayatsu and Utika [360]).

100 µg. of sRNA (devoid of bound amino acid) was

incubated with 1 μmole of ATP, 200 mμmoles of CTP, 10 μmoles of KCl, 2 μmoles of $MgCl_2$, 10μmoles of Tris-HCl buffer pH 7.6, 0.5 - 1.0 units (1 unit = A_{280} x volume in ml.) of ligase either 10 mμmoles of ^{14}C - labelled algal protein hydrolysate or 10 - 20 mμmoles of a ^{3}H- or ^{14}C-labelled amino acid, with 20 mumoles of each of the following amino acids: Glycine, Alanine, Leucine, Isoleucine, Serine, Threonine, Valine, Lysine, Arginine, Glutamic acid, Aspartic acid, Glutamine, Asparagine, Cysteine, Methionine, Phenylalanine, Tyrosine, Tryptophan, Proline and Histidine.

This mixture, in a total volume of 0.25 ml., was incubated at 37° for 17 minutes, precipitated onto a filter disk and washed with 10% w/v trichloracetic acid containing unlabelled amino acid at 100 μg./ml. prior to radioactivity measurement. Alternatively, the incubated mixture was diluted with 0.2 ml. 0.3M KCl and shaken with an equal volume of 90% phenol for 2 - 3 minutes. 0.4 ml. 0.5M sodium acetate buffer pH 5.1 was added and shaking continued for 5 minutes. After centrifugation at 10,000g for 5 minutes, the aqueous layer was removed and precipitated with 2% sodium acetate and 67% ethanol. The precipitate was washed with ethanol and ether and dried in air. The final dried

material was dissolved in a small volume of 0.1M sodium acetate buffer pH 5.5 containing 0.005M EDTA and stored at -70°.

11.
(c) Treatment of Aminoacyl tRNA with T1 RNase.

500 µg. of aminoacyl tRNA were incubated with 500 units of T1 RNase for various times at 37° in 0.1M sodium acetate buffer pH 5.5. This pH is far from the enzyme's optimum, (pH 7.5) but serves to retain the integrity of the aminoacyl tRNA.

The digest was applied in small volume to a DEAE-cellulose column (10cm. x 1cm.), washed with 0.01M ammonium formate pH 5.5 or pH 7.0., and eluted with a gradient (0.01M to 0.5M) of ammonium formate. After measurement of absorbancy at 260 mµ, each fraction was evaporated to dryness at 150° in scintillation vials and assayed for radioactivity by adding 10 ml. of Toluene-based scintillation fluid to each vial.

SECTION III.

RESULTS.

HERPES SIMPLEX DNA.

1. Virus Purification.

Ten 80 oz. bottles of BHK21(C13) cells were infected with Herpes Simplex virus strain a and the resulting virus harvested (Methods Sect. I(b)) after 42 hours. The virus yield was freed of gross cellular contamination by the differential centrifugation procedure (Methods Sect. I(c)) and divided into two equal parts.

One half was applied to and eluted from a column of Brushite (Methods Sect. 2(a)), the virus appearing in two peaks corresponding to elution with 0.4M potassium phosphate buffer pH7 (Fraction 1) and 1.0M potassium phosphate buffer pH7 (Fraction 2) respectively. Concomitantly, the remainder of the crude virus yield was treated with pancreatic RNase and DNase I, washed by centrifugation and resuspended (Fraction 3) (Methods Sect. I(d)). All three fractions were dialysed against SSC, and DNA was extracted from them by the method of Russell (Methods Sect. 3(b)).

Yields of DNA were measured by the method of Ceriotti (Methods Sect. 6(b)) and related to yield of virus; the extent of contamination of viral DNA with host DNA was established by CsCl equilibrium

F I G U R E 21.

Densitometer tracing of the positions of Herpes Simplex virus DNA and $\left[{}^{15}N\text{-}{}^{2}H\right]$ DNA from Escherichia coli after CsCl equilibrium centrifugation in the Spinco model E ultracentrifuge.

Herpes Simplex virus DNA = 1,726 gm./ml.

Escherichia coli DNA = 1,747 gm./ml.

FIGURE 21

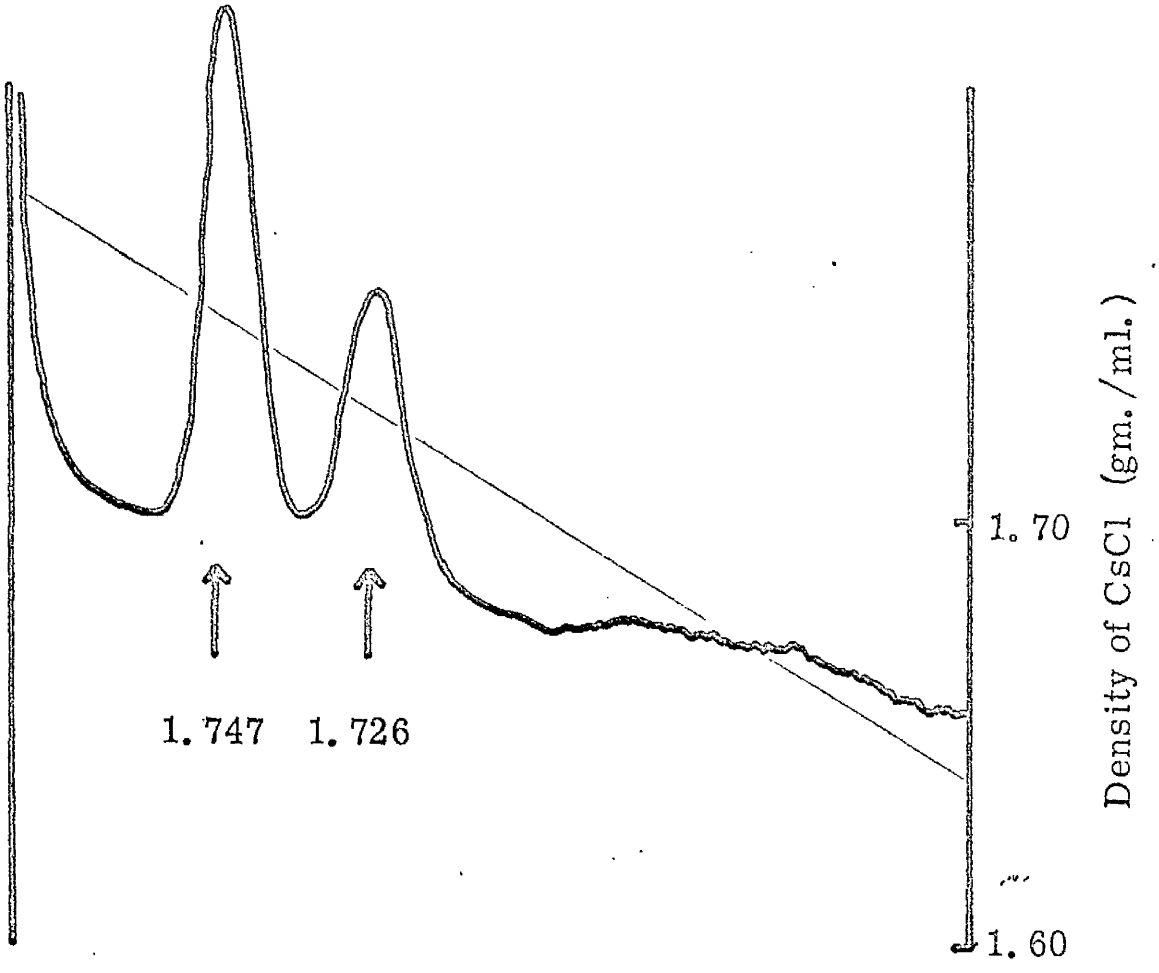


TABLE 2.

Evaluation of Herpes Simplex DNA produced by different methods. Protein was measured by the method of Lowry et.al.[345] and RNA by the Orcinol proceduro (methods sect.6c).

TABLE 2

Herpes DNA prepared by	Absorbancy mμ 260	280	Protein content per μg. DNA	RNA content per μg. DNA	product integrity	ratio $\frac{260}{280}$
Phenol method	0.49	0.28	not detected	0.02μg.	would not "spool"	1.75
Marmur method	0.58	0.36	0.07ug.	0.03μg.	"spooled" characteristically	1.65

density gradient centrifugation in the Spinco Model E ultracentrifuge (Fig. 21). (Methods Sect. 8(b).). However, infectivity measurements and virus particle counts (Russell & Crawford [191]) revealed that the Brushite column procedure reduced the total virus population by 50 - 60%, while the DNase and RNase procedure incurred no such loss: hence this method was used in all further work.

2. Preparation of DNA.

A batch of virus prepared as in the last experiment and purified by DNase and RNase treatment was divided into two parts, and respectively subjected to (a) the Phenol method (Methods Sect. 3(c)) and (b) the method of Marmur (Methods Sect. 3(a)) for preparation of DNA.

The resulting DNAs were examined using the following criteria: the absorbancy at 260 mμ (Table 2), which gives an approximate measure of the quantity of DNA present (45μg DNA = one A_{260} unit) and the ratio of absorbancy at 260mμ to that at 280mμ, (Table 2), which provides an estimate of the contamination of the preparation by protein. The theoretical ratio $\frac{A_{260}}{A_{280}}$ for protein-free DNA is approximately 1.8 to

2.0 under the conditions employed. The finding by this method that the DNA prepared by the Phenol method contains less protein than that prepared by the method of Marmur (Marmur [284]) is confirmed (Table 2) by the quantitative procedure for estimation of protein (Methods Sect. 6(a)).

The RNA content of both preparations of DNA was assayed using the orcinol method (Methods Sect. 6(c)) (Table 2), while an approximate estimate of the molecular integrity of the products was obtained by their ability to "spool" onto a glass rod (Table 2).

From these results, it can be seen that the phenol method is more efficient in removing protein from the viral DNA, but, in this process, appears to have a degradative effect on the DNA: the extent of contamination of both preparations revealed in the orcinol reaction with RNA is small, and this was probably due to cross reaction between the DNA and the reagents used (Mejbaum [278]). This method of estimation of RNA was therefore not considered completely quantitative.

On the basis of these results, a method of Herpes Simplex virus DNA preparation was devised which employed features taken from both of the above methods:

TABLE 3.

Examination of Herpes Simplex DNA isolated using the "Modified Marmur method".

DNA and RNA were estimated using the method of Fleck & Munro [350] and protein estimations were carried out using the method of Lowry et.al. [345]

TABLE 3

DNA µg.	RNA µg.	Protein µg.	S value	Ratio $\frac{260}{280}$
350	10	10	21	1.77

this was named the "modified Marmur" method. (Methods Sect. 3(d)).

A sample of purified herpes virus was treated using the "modified Marmur" DNA preparative method, and the product assayed for DNA and RNA content using the modified Schmidt and Thannhauser technique (Fleck & Munro [350]), and for protein content by the method of Lowry, Rosebrough, Farr and Randall (Methods Sects. 6(d) & 6(a)). The results (Table 3) show the method to be satisfactory from the point of view of minimal contamination with RNA and protein. The product DNA was obtained as a "spool", and had a sedimentation coefficient of 21S. The method therefore provided DNA of satisfactory purity and molecular size. Isolated DNA was routinely stored at 4° in SSC and in the presence of a small amount of $CHCl_3$.

This method was developed using Herpes Simplex virus DNA, but DNA from mammalian cells was prepared in the same manner with satisfactory results: 70% to 80% yields of DNA were routinely obtained in all cases.

The protein separated out in the course of the preparation of DNA was washed, dispersed with the aid of ultrasonic vibrations, dissolved in dilute alkali

and assayed for DNA content: this was found to be in the region of 5%.

Tests for RNase activity were carried out both on DNA prepared by the standard method and on DNA which had been isolated after treatment with Bentonite at the end of the RNase digestion step: in neither sample was there detectable RNase activity.

3. CHARACTERISTICS OF ISOLATED DNA.

(a) Biological Activity.

Herpes virus DNA is not per se capable of infecting BHK21(C13) cells, but it could possibly become infective if a small quantity of intact virus is added to it.

Two strains of Herpes Simplex virus exist which lend themselves to investigation of this phenomenon. They give rise to two morphologically distinct plaque types on infection of a host cell monolayer: these are the mP and MP strains. DNA isolated from purified MP Herpes Simplex virus was added to particles of mP Herpes Simplex virus (1µg/10^4 particles) and the mixture used to infect BHK21(C13) cell monolayers at a multiplicity of exposure of 10 plaque-forming units/cell. The cell sheets were examined after 3 - 4

days and plaques were classified and counted. Plaques of both mP and MP types were observed suggesting, in a preliminary way, that this DNA preparation has biological activity. However, the result is not accepted as unequivocal proof of the transforming ability of Herpes Simplex virus DNA.

(b) Buoyant Density of DNA in CsCl solutions.

The buoyant density of a DNA is determined by its base composition and its double or single stranded nature. Purified Herpes Simplex virus DNA was dissolved in CsCl solution (final density = 1.70 gm/ml.) and centrifuged in the Spinco Model E ultracentrifuge. (Methods Sect. 8(b)). 4µg of $\left[^{15}N - ^{2}H\right]$ DNA from Escherichia coli was present to act as a density marker. At equilibrium, the positions of the DNAs in the CsCl gradient were determined by their ultraviolet absorption. A densitometer trace of the resulting photographic negative is shown in Fig. 21. The buoyant density (ρ) of an unknown DNA sample may be calculated using the method of (Sueoka [277]) where

$$\rho = \rho_o + 4.2\omega^2 (r^2 - r_o^2) \times 10^{-10}$$

where ρ_o = density of reference

ω = angular velocity

r_o = radius of reference peak

F I G U R E 22.

Densitometer tracing of the positions of Herpes Simplex DNA and BHK21 (C13) (host) DNA after CsCl equilibrium centrifugation in the Spinco model E ultracentrifuge.

F I G U R E 23.

A tracing identical to the above showing three DNAs:

Herpes Simplex virus DNA = 1,727 gm./ml.

BHK21 (C13) DNA = 1,697 gm./ml.

$\left[{}^{15}N\text{-}{}^{2}H\right]$ *Escherichia coli* DNA = 1,748 gm./ml.

FIGURE 22

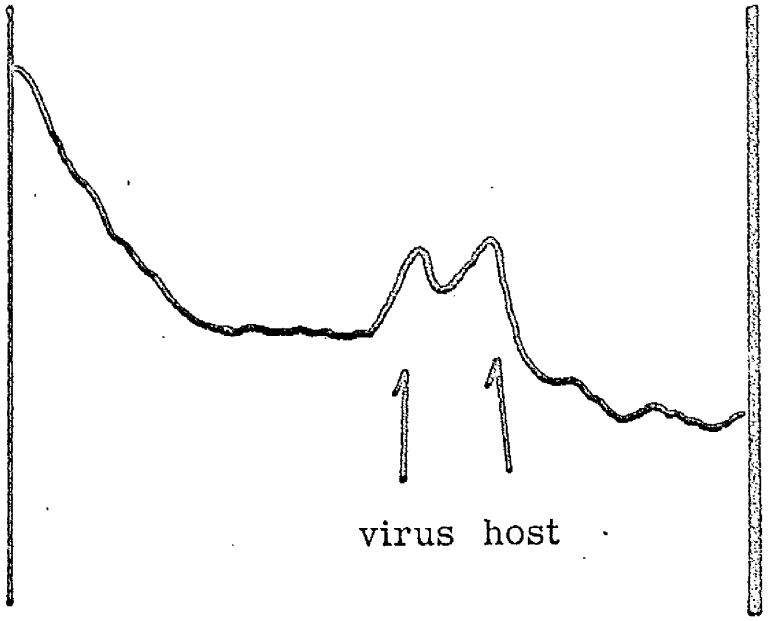


FIGURE 23

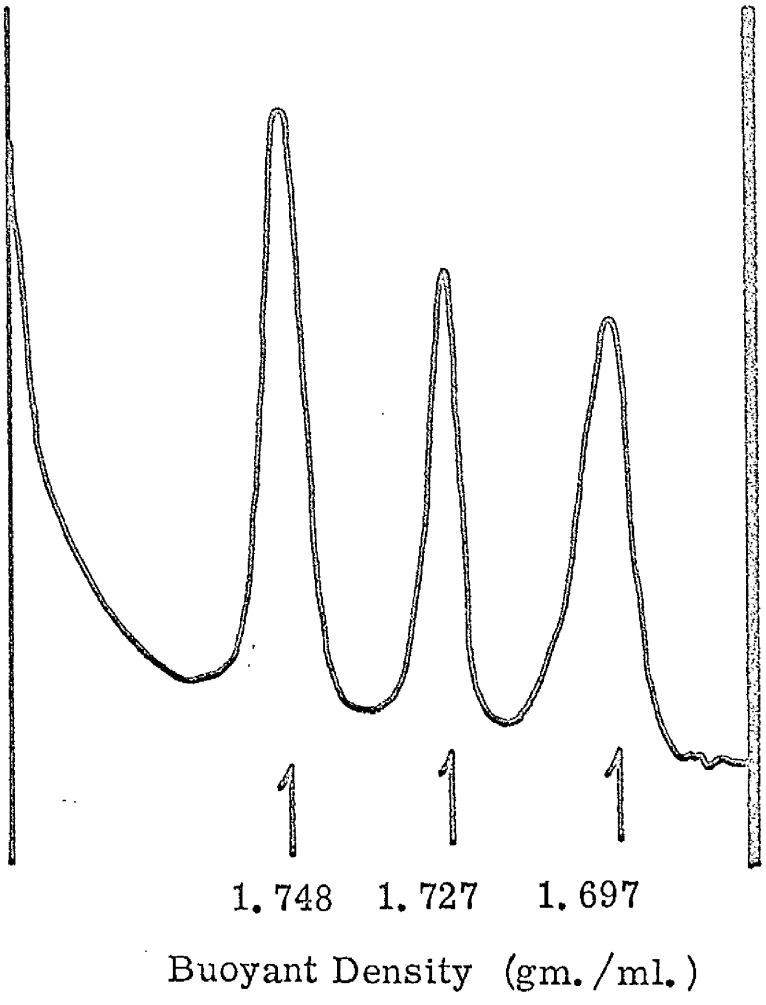


TABLE 4.

Buoyant densities and base compositions of Herpes Simplex virus and BHK21 (C13) cell DNAs.

Base compositions were calculated from buoyant densities by the method of Sueoka *et.al.*[279].

TABLE 6.

Evaluation of two methods of removing RNA from a preparation of Herpes Simplex virus DNA.

TABLE 4

DNA source	Buoyant density gm./ml.	Percentage Guanine plus Cytosine
Herpes Simplex Virus	1.726	68
BHK 21 (C13) cells	1.702	41

TABLE 5

Treatment	Yield of DNA (μg.)	Contaminating RNA
Ribonuclease	208	less than 2%
Iso propanol	122	less than 2%

r = radius of peak

The buoyant density of Herpes Simplex virus DNA was thus calculated to be 1.725 gm/ml. This is in good agreement with the work of (Russell & Crawford [191]), and, if the base composition of the DNA is determined by the method of (Sueoka et.al. [279]), the value obtained is 68% (G plus C). The identity is $\rho = 1.660 + 0.098$ (G plus C).

An identical CsCl gradient analysis of DNA extracted from partially purified Herpes Simplex virus is shown in Fig. 22. It can be seen that there are two DNAs (from virus and host) which are clearly separable using this procedure. A more quantitative assessment of the buoyant density relationships of these two DNAs was carried out in the above system using a mixture of purified host (BHK21(C13)) DNA, Herpes Simplex virus DNA and the Escherichia coli marker DNA. The result (Fig. 23) illustrates the excellent separation of the three DNAs. Table 4 gives the buoyant densities and base compositions of Herpes Simplex virus and BHK21(C13) DNAs calculated from the analytical data given above.

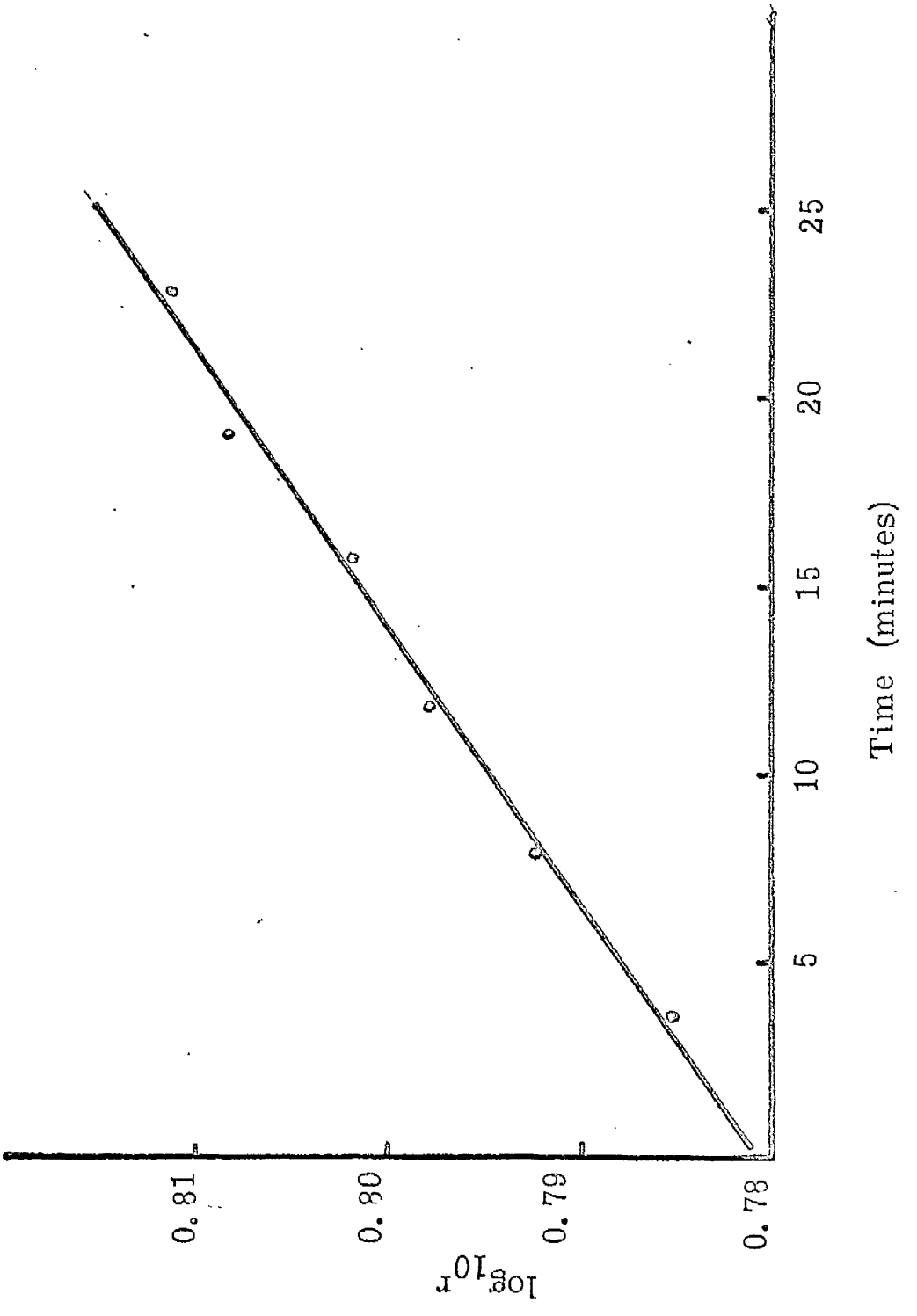
3.

F I G U R E 24.

The logarithm of the distance of the boundary of DNA sedimenting in the Spinco model E ultracentrifuge from the centre of rotation of the rotor as a function of time.

The slope of the curve = $\frac{d \log r}{dT}$, and this is substituted in the Svedberg equation.

FIGURE 24



F I G U R E 25.

The effect of heating on the absorbancy at 260mμ of two DNA samples:

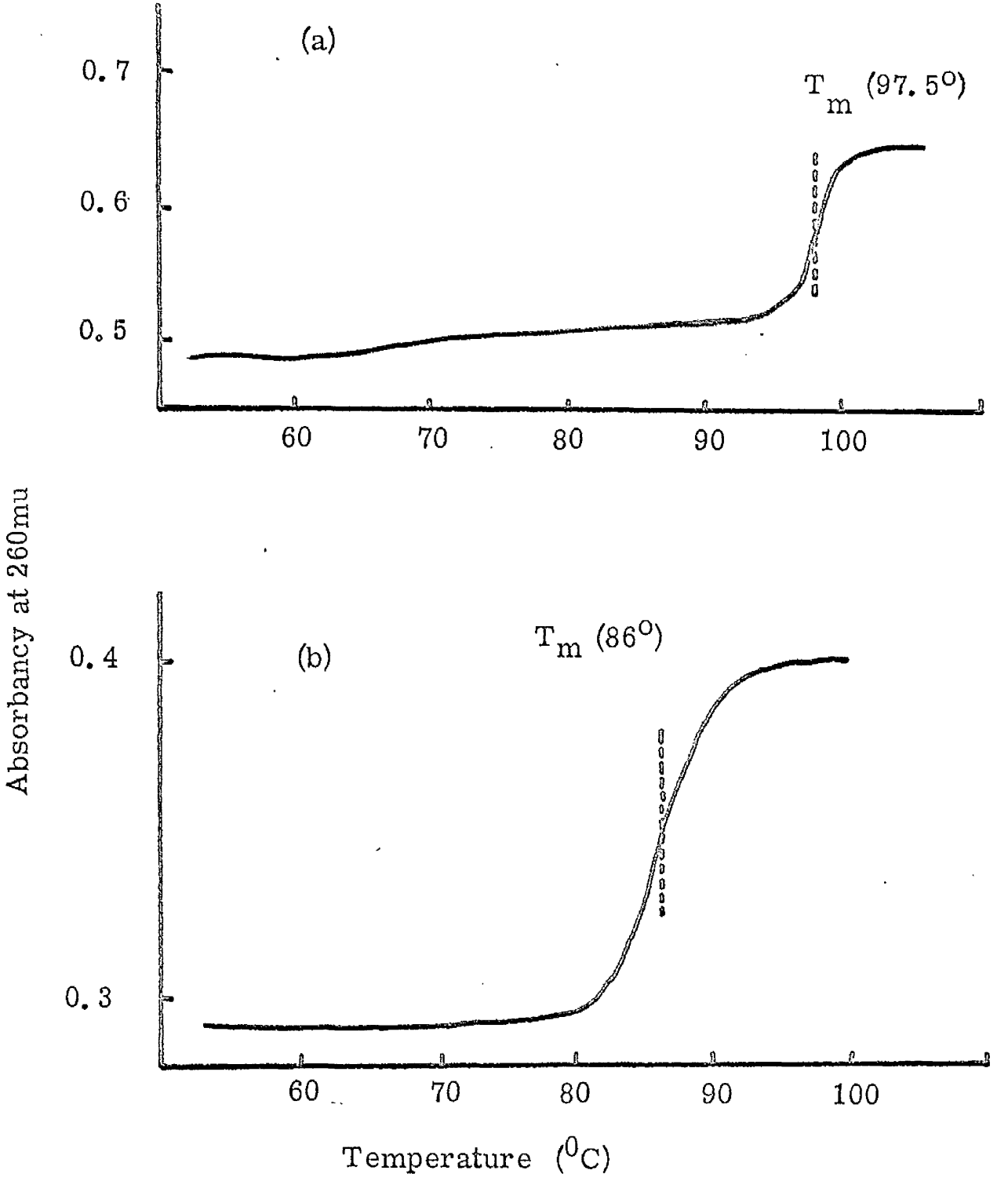
(a) Herpes Simplex virus DNA

(b) BHK21 (C13) DNA

The "melting temperatures" are (a) 97.5°, (b) 86°.

The solvent used was SSC.

FIGURE 25


(a)
0.7
0.6
0.5
T_m (97.5°)
60
70
80
90
100
(b)
0.4
0.3
T_m (86°)
60
70
80
90
100
Absorbancy at 260mu
Temperature (°C)

(c) Size of DNA product.

Purified Herpes Simplex virus DNA (Methods Sect. 3(d)) was centrifuged in 0.15M NaCl at 44,770 r.p.m. in the Spinco Model E Analytical ultracentrifuge. At 4 minute intervals after initiating the centrifugation, ultraviolet photographs of the boundary of sedimenting DNA were taken and from these was estimated the distance of the boundary from the centre of rotation of the rotor. The logarithms of these values as a function of time are shown in Fig. 24. The slope of this function is $\frac{d\log_{10}r}{dT}$, and, inserting the value for this slope in the Svedberg equation (Svedberg & Nicols [352]) $S = \frac{1}{w^2} \times \frac{d\log r}{dT}$, we find that the Herpes Simplex virus DNA sample has a sedimentation coefficient of 24S. The calculation of the m.w. of large DNA molecules from sedimentation coefficients is subject to some uncertainty (Russell [384]) but this may be estimated (Rubenstein et al. [280]) to be 20×10^6, representing a fragment of the intact DNA molecule of the virus (Russell & Crawford [191]).

3.

(d) Melting temperature (Tm) determinations.

Fig. 25 shows the effect of heating on the

absorbancy at 260mμ of (a) a sample of DNA from purified Herpes Simplex virus and (b) a sample of BHK21 (C13) cell DNA (Methods Sect. 3(d)). It can be seen that there is in both cases a marked increase in A_{260} (a) between 93° and 102° and (b) between 81° and 91.5° consistent with the collapse of a double-stranded structure (Marmur & Doty [281]). The temperature at the mid-point of the increase in absorbancy (Tm or denaturation temperature) is, for Herpes Simplex virus 97.5°, and for BHK21 (C13) 86°. (Marmur & Doty [282]) have expressed the relationship between the Tm (under specified ionic conditions) and the base composition of the DNA: this is:-

$$Tm = 69.3 + 0.41\ (G + C)$$

According to this calculation, the DNA of Herpes Simplex virus has a base composition of 69%(G + C,) and that of BHK21 (C13) cells 41%,(G + C.)

When the above procedure was carried out on a mixture of Herpes Simplex virus DNA and HEp2 DNA in $\frac{1}{10}$ SSC (Marmur & Doty [282]) the profiles shown in Fig. 26 were obtained. The Tm for Herpes Simplex virus falls, under these conditions, to 84.5°, and that for HEp2 DNA, which is very similar to BHK21 (C13) DNA in its base composition (Morrison [351]) is 70°. Here also, a sharp

thermal transition can be observed in both DNA samples, consistent with their double-stranded nature. Recently (Schildkraut & Lifson [283]) have derived the relationship

$$Tm = 16.6 (\log M) + 0.41 (GC) + 81.5$$

where M = molarity of solvent

Applying the values obtained for the Tm of Herpes Simplex virus and HEp2DNA in 1/10 SSC, theGplus C content of these DNAs becomes respectively 78% and 44%. This value for HEp2 DNA is acceptable (Morrison [351]) but that for Herpes Simplex virus does not agree with previous findings. The reason for this discrepancy may be that the mathematical relationship was constructed for bacterial DNAs whose G plus C content was similar to that of Herpes Simplex virus, but whose size and structure may be different. Also, the relationship may deviate markedly beyond any upper limit.

3.

(e) Complete separation of the two strands of DNA.

In the course of investigations designed to determine the Tms for Herpes Simplex virus DNA and host cell DNA, it was observed that, in the case of the viral DNA, renaturation of the heat-dissociated strands

F I G U R E 26.

The effect of heating on the absorbancy at 260mμ of a mixture of Herpes Simplex virus and BHK21 (C13) DNAs.

The "melting temperatures" are 84° and 70° for virus and host respectively. The solvent used was 1/10 SSC.

F I G U R E 27.

The influence of salt concentration on the thermal denaturation of DNA.

Herpes Simplex virus and BHK21 (C13) DNAs were heated to 100° for 10 minutes in the presence of several concentrations of SSC, then cooled rapidly in an ice-bath. The hyperchromicity observed was taken as a measure of the extent of denaturation of the DNAs.

FIGURE 26

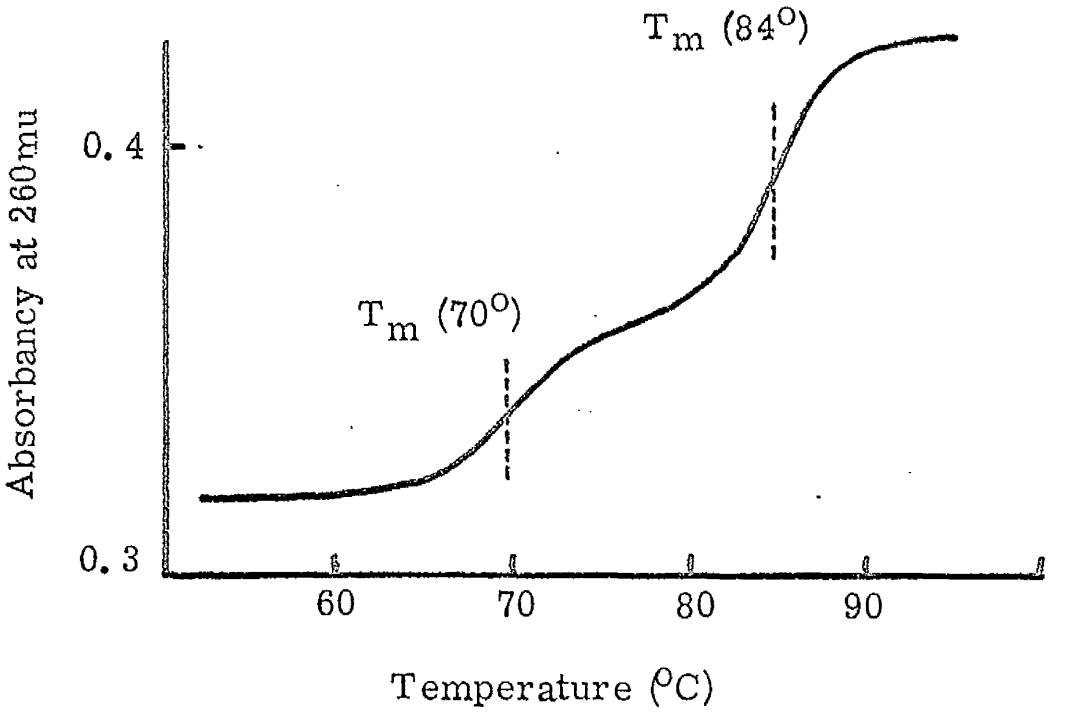
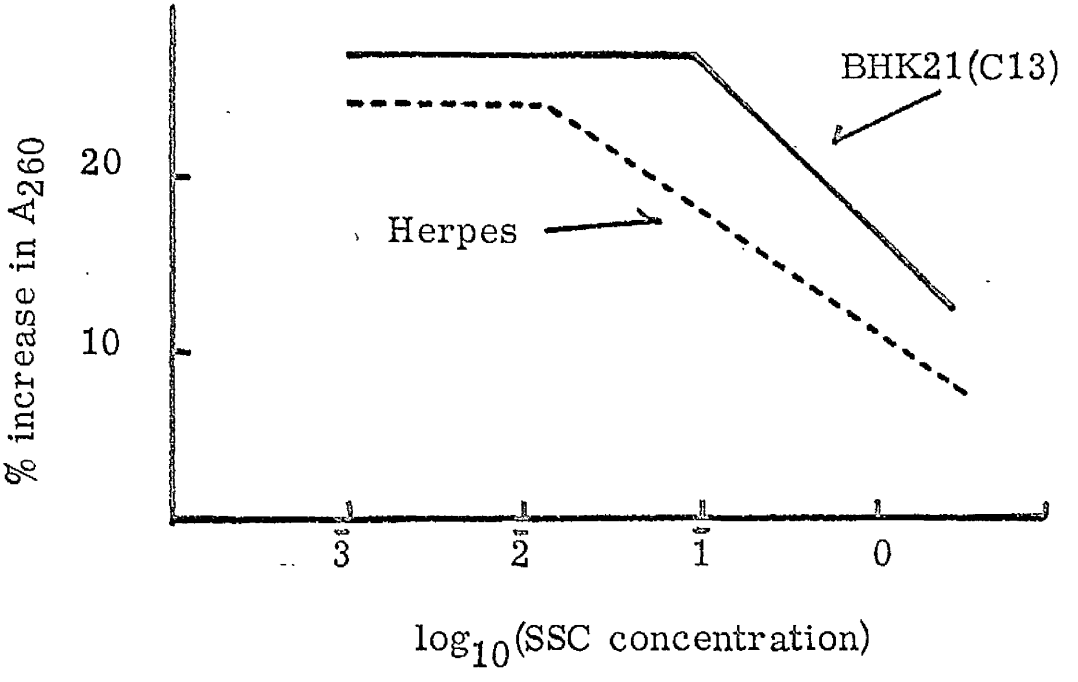


FIGURE 27



took place spontaneously on cooling in SSC or 1/10 SSC. BHK21 (C13) DNA behaved similarly in SSC. In view of the fact that irreversibly heat-denatured DNA would be required for DNA-RNA hybridisation studies, samples of Herpes Simplex virus DNA and BHK21 (C13) DNA were heated at 100°C in the presence of a range of concentrations of SSC. After 10 minutes, the samples were cooled rapidly by immersion in an ice-water bath and the increase in absorbancy at 260mμ resulting from the heating and cooling process was taken as a measure of the degree of irreversible denaturation of the DNAs.

Fig. 27 relates this increase in absorbancy to the logarithm of SSC concentration used. It is evident that irreversible denaturation of BHK21 (C13) DNA can be achieved using 1/10 SSC as solvent, but for Herpes Simplex DNA, concentrations of 1/50 SSC or lower must be used.

3.

(f) <u>Effect of addition of salt to a solution of denatured Herpes Simplex virus DNA.</u>

It is important, once more from the point of view of hybridisation experiments with RNA, to quantitate the effect of increasing the salt

concentration of a sample of heat-denatured Herpes Simplex virus DNA. A sample of Herpes Simplex virus DNA was heated to 100°C for 15 minutes in 1/50 SSC and cooled in ice, the resulting hyperchromicity being 24% (Fig. 27). Addition of 10X SSC to a final concentration of 2 x SSC was observed to have no effect on the absorbancy increase at 260mμ (after correction for the dilution involved). From this it is inferred that no renaturation of Herpes Simplex virus DNA takes place under these conditions.

4. PREPARATION OF HERPES SIMPLEX VIRUS DNA. FURTHER CONSIDERATIONS.

(a) The influence of added protein and the use of Arcton in preparation of DNA from Herpes Simplex virus.

DNA was extracted from purified Herpes Simplex virus in one of the following ways (i) modified Marmur method (Methods Sect. 3(d)) (ii) modified Marmur method after the addition of bovine serum albumin to 2mg./ml. (iii) modified Marmur method using Arcton 113 (trichlorotribromoethane) (iv) as (ii) using Arcton in place of $CHCl_3$ and *iso* amyl alcohol. It was felt that protein might serve to make the removal of Herpes Simplex virus coat protein more effective, and Arcton is a well-known deproteinising agent. The

product DNAs were examined for ability to be "spooled", for protein content and for total yield (Table 5). The addition of Bovine Serum Albumin had no significant effect on any of the parameters tested. Arcton appeared to be more useful in the removal of protein than was $CHCl_3$ and iso amyl alcohol, but DNA produced by this method could not be "spooled", suggesting that Arcton has a degradative action on DNA. This possibility was tested by treating the "spooled" DNA prepared by the modified Marmur method for 15 minutes with Arcton at room temperature. As a result of this treatment, the DNA lost its ability to be "spooled".

Neither protein nor Arcton was employed subsequently in the preparation of DNA.

4.

(b) Use of iso-propanol.

Addition of iso-propanol to a solution of DNA and RNA, with stirring, results in the preferential precipitation of DNA as a "spool" on the stirring rod (Marmur [284]). A batch of Herpes Simplex virus was deproteinised exhaustively with $CHCl_3$ and iso amyl alcohol as in the modified Marmur method (Methods Sect. 3(d)). One half was treated with RNase (15µg./ml.

TABLE 4.

The effect of added protein and the influence of 'Arcton' on the isolation of Herpes Simplex virus DNA.

The protein used was bovine serum albumin (B.S.A.)

TABLE 5

Extraction technique for DNA	Ratio $\frac{260}{280}$	Yield µg.	Ability to "spool"
"Modified Marmur"	1.95	285	+
"Modified Marmur" + B.S.A.	1.94	277	+
"Modified Marmur" + Aroton	2.00	249	-
"Modified Marmur" + B.S.A. + Aroton	2.00	278	-

at 37° for 30 min.), deproteinised and the DNA precipitated with ethanol; the remainder was stirred during the addition of iso propanol and the DNA "spool" was removed from the glass rod. Both DNA samples were dissolved in SSC and assayed for DNA (Results Sect. 2) and RNA (Methods Sect 6(c)) (Table 6). Both RNA-removing procedures are effective, but the yield of DNA obtained using iso propanol is markedly lower than that found with RNase treatment. The very low level of RNA contamination can be ascribed to the fact that in the initial deproteinising stages, the viral DNA had been spooled from an ethanol solution; this procedure tends to free DNA from any RNA present. Iso-propanol was not subsequently used in purification of DNA.

5. FRACTIONATION OF DNA.

(a) MAK columns.

The possibility arose that a rapid method of preparation of pure Herpes Simplex virus DNA might be found in extraction of DNA from crude infected cell material (Methods Sect. 3(c)) followed by separation of host and virus DNA on MAK columns (Methods Sect. 2(b)). Such a preparation of crude DNA (125µg.) was

F I G U R E 28.

Fractionation of DNA prepared from Herpes Simplex virus - infected BHK21 (C13) cells on a column of MAK.

The elution was carried out using batches of increasing salt concentration as shown, and took place at 20°.

F I G U R E 29.

MAK column fractionation of a ten times greater amount of the above DNAs.

The two major peaks (I & II) were assayed for DNA content by the method of Ceriotti (Methods sect.6b) and for RNA content by the Orcinol procedure (Methods sect.6c).

The elution, at 20°, was carried out using batches of increasing salt concentration as shown.

DNA present

RNA present

FIGURE 28

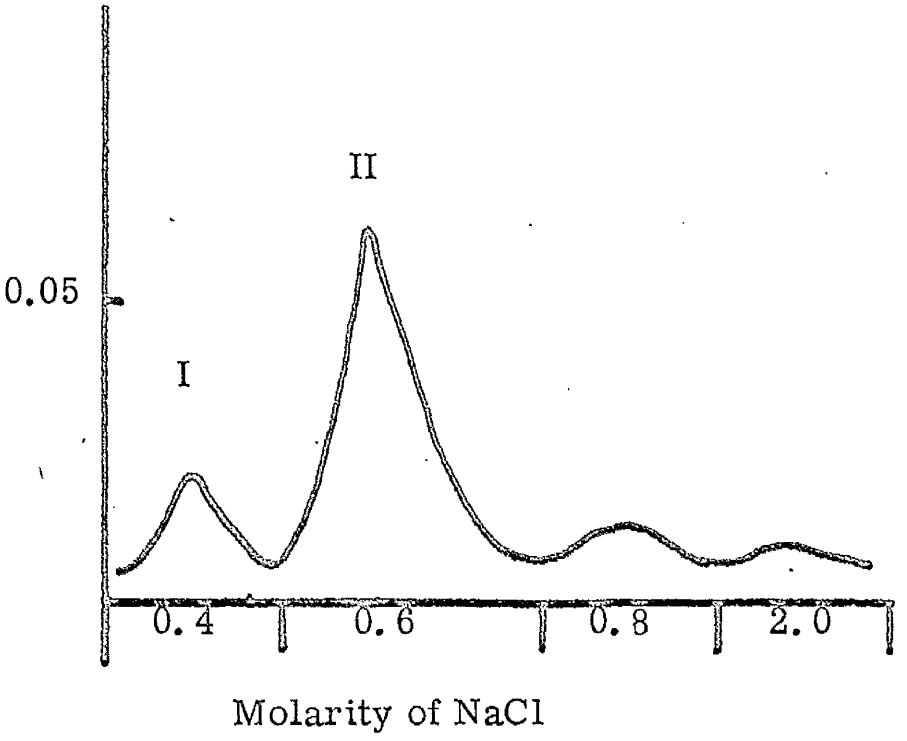
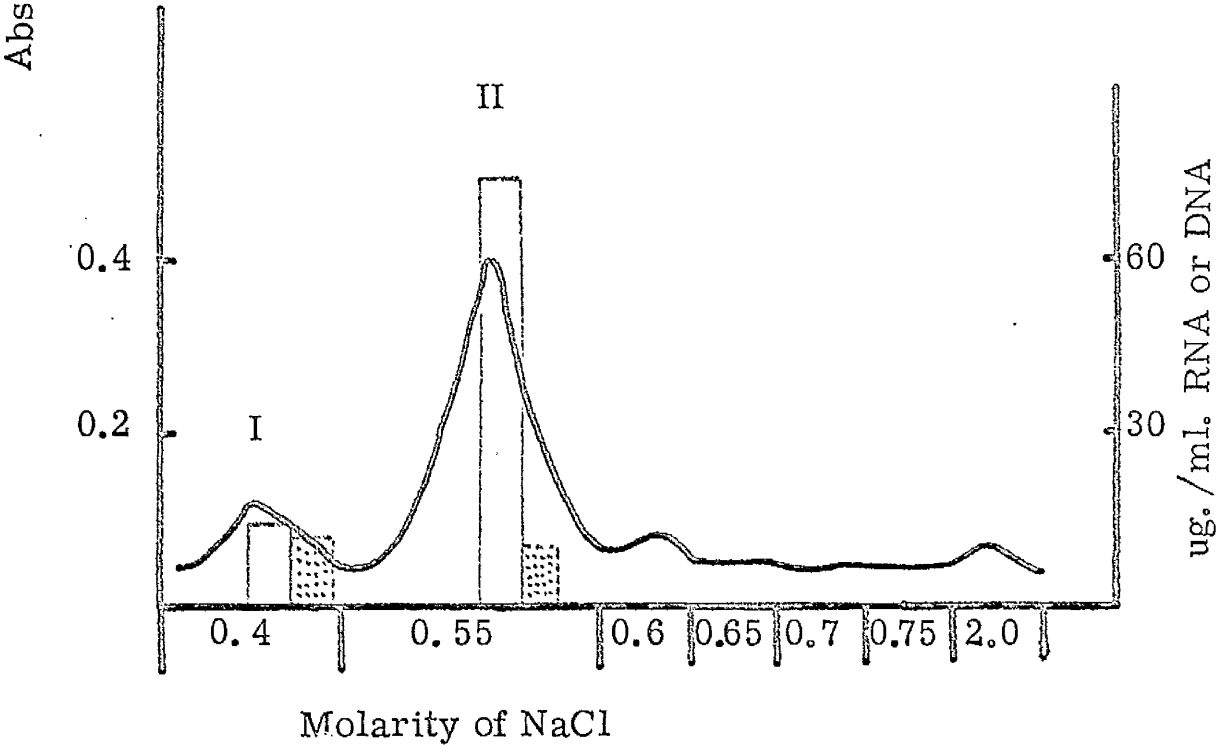


FIGURE 29



applied to a 2 ml. column of MAK and eluted with 5 ml. batches of increasing concentrations of NaCl in Na_2HPO_4/NaH_2PO_4 buffer pH 6.7. The pattern of elution (Fig. 28) shows two major peaks of absorbancy at 260mμ which elute at 0.4M and 0.6M NaCl respectively. It was tempting to regard these peaks as virus DNA (higher G plus C) and host DNA respectively but the first peaks emerged from the column too early to be DNA, and the separation of the peaks was too wide to be explained by the relatively fine difference between host and virus DNA. Moreover this experiment was carried out on a pilot scale, and the column effluent was too dilute for accurate chemical analysis. The recovery of DNA from the column was 100%. The fractionation was repeated scaled up ten times, and the DNA eluted stepwise with the following concentrations of NaCl in sodium phosphate buffer, pH6.7:0.4M; 0.55M; 0.60M; 0.65M; 0.70M; 0.75M; 2.00M. The elution pattern (Fig. 29) corresponds well with the pattern previously obtained (Fig. 28). Estimation of RNA (Methods Sect. 6(c)) and DNA (Methods Sect. 6(b)) in peak fractions indicated that peak I consisted of DNA and RNA, while peak II contained only DNA. This distribution is almost certainly due to the degraded nature of the nucleic acid

F I G U R E 30.

The fractionation of calf thymus DNA on the MAK column.

Elution was achieved at 20° using batches of successively greater salt concentration.

F I G U R E 31.

Fractionation of DNA isolated from Herpes Simplex virus-infected BHK21 (C13) cells on a column of MAK.

The DNA was prepared using the "Modified Marmur" technique (Methods sect.3d) and was eluted from the column at 20° using the salt concentrations shown.

FIGURE 30

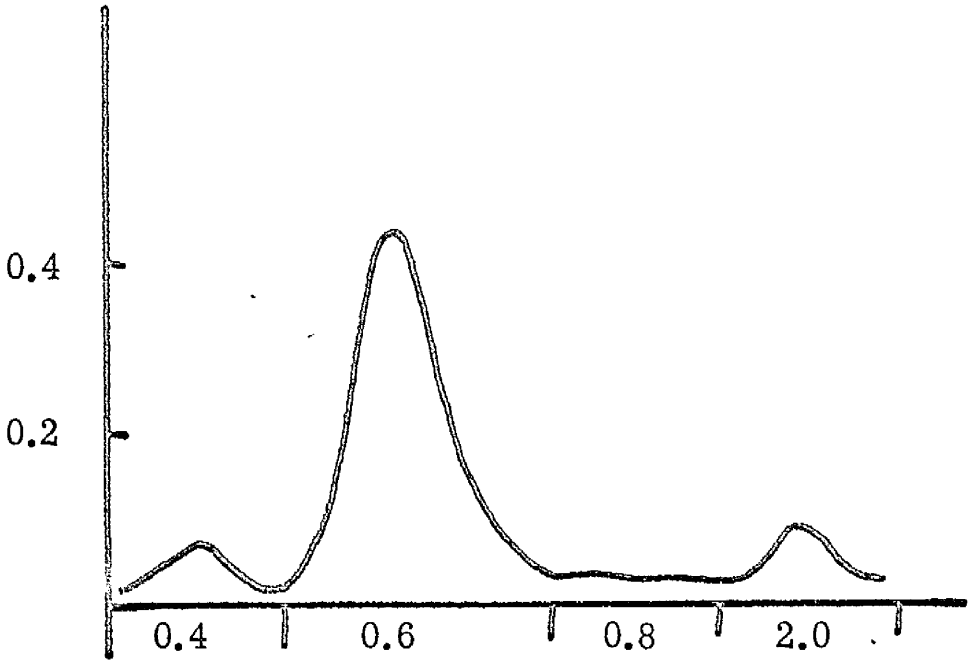
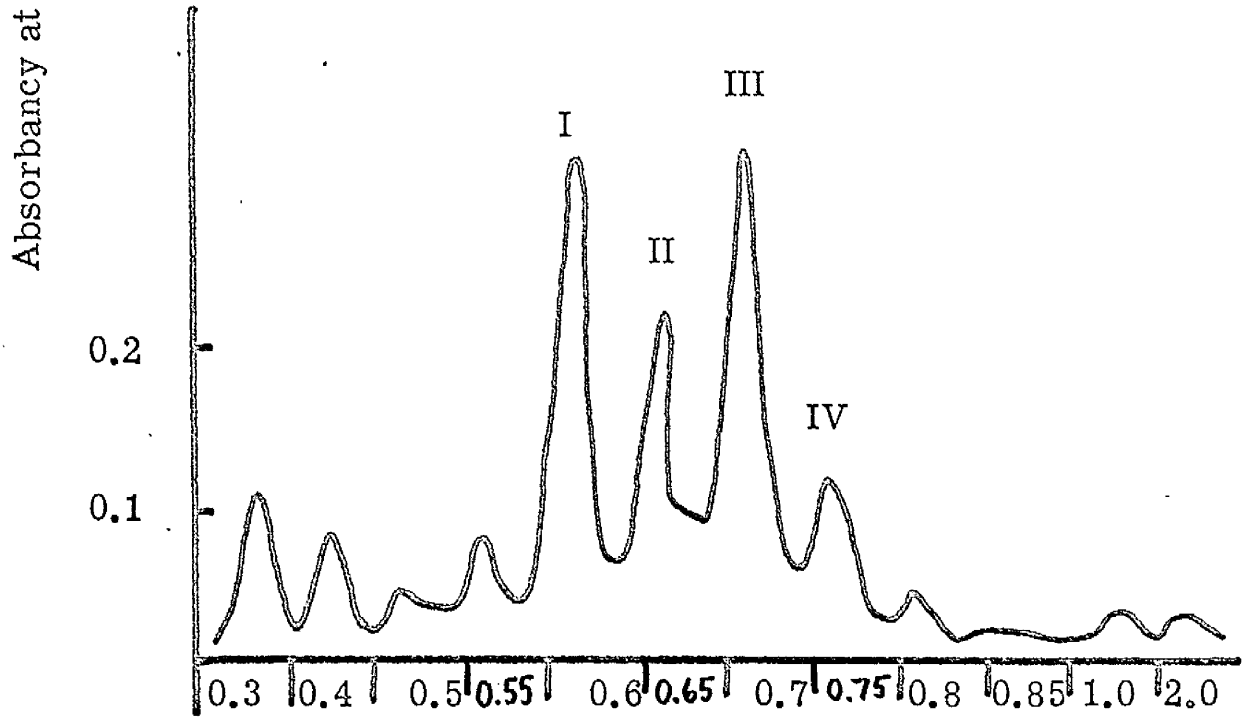


FIGURE 31



preparation: the finding of high molecular weight RNA is surprising, since the DNA preparation was treated with RNase during isolation. However, residual phenol may have affected the RNase action.

A sample of high molecular weight DNA from calf thymus was obtained from laboratory stock and was subjected to fractionation in the above way. (Fig. 30). Most of it eluted from MAK as a single band at 0.6M corresponding to double-stranded mammalian DNA (Yoshikawa-Fukada et.al. [331]); some single-stranded material was also evident at 2M NaCl (Sueoka & Cheng [59]).

In the light of this work fresh infected cell material was carefully fractionated for DNA, using the modified Marmur technique (Methods Sect. 3(d)) and applied to a column of MAK prepared by the technique of Hayashi, Hayashi and Spiegelman (Methods Sect. 2(b) (i)).

Several fractions were eluted from the column using the elution technique above (Fig. 31). Fraction I and III were examined by CsCl density gradient ultracentrifugation in the Spinco Model E (Methods Sect. 8(b)) and both were found to contain virus and host DNA, the latter in abundance.

The DNA fractions eluting at 0.65M, 0.70M, 0.75M NaCl were heated slowly to 100°C in the melting

F I G U R E 32.

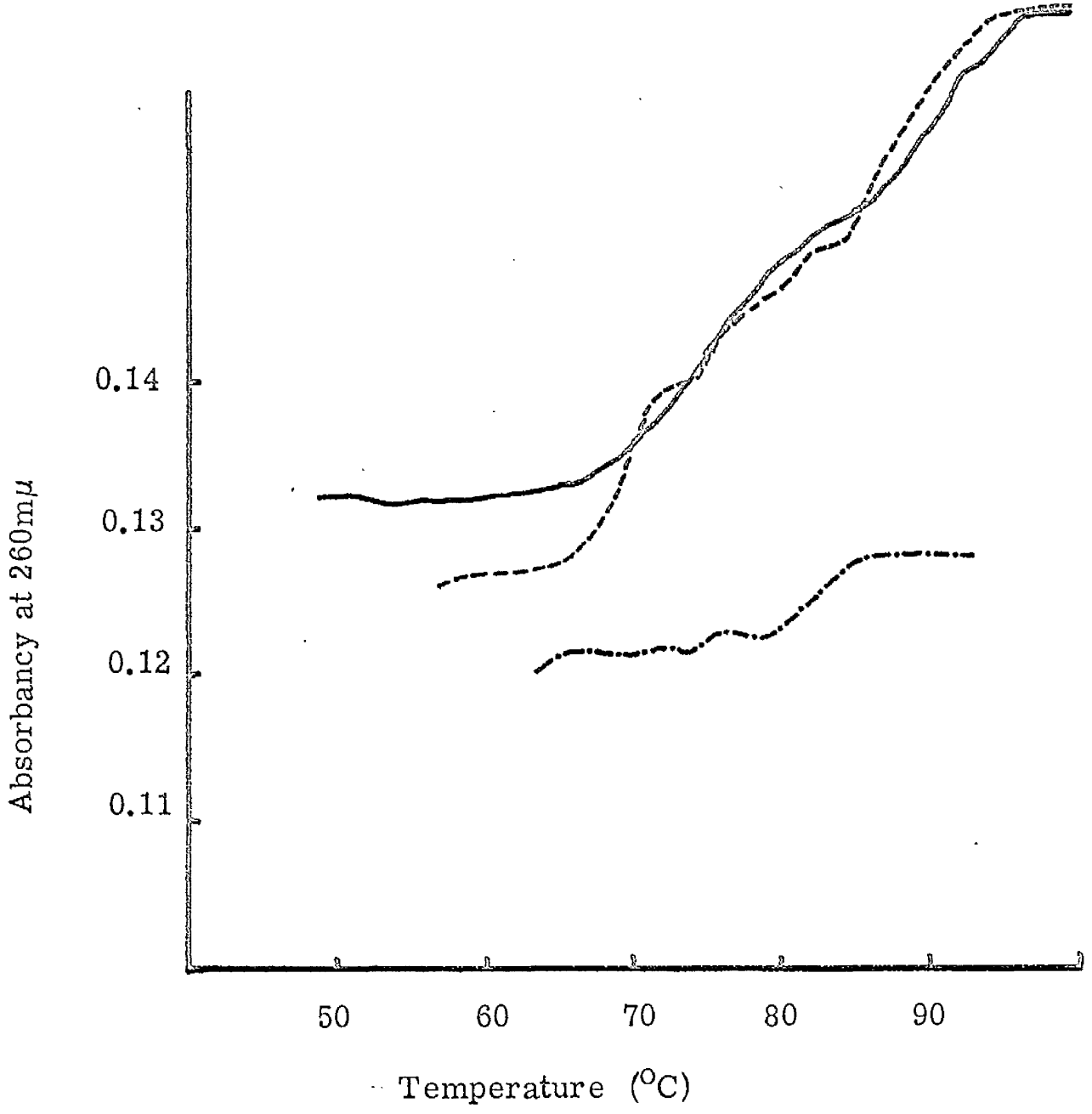
The effect of heating on the absorbancy at 260mu of DNA fractions obtained from Figure 31.

———— 0.65M fraction

– – – – 0.70M fraction

—.—.— 0.75M fraction

FIGURE 32


0.14
0.13
0.12
0.11
Absorbancy at 260mμ
50
60
70
80
90
Temperature (°C)

temperature apparatus (Results Sect. 3(d)). It was hoped that the much higher Tm of the viral DNA would allow it to be distinguished from the host material, but all of the DNA fractions obtained gave the broad temperature-absorbancy profile obtained using either host DNA or a host-virus DNA mixture. (Fig. 32: cf also Fig. 26).

From these results it seems evident that the fractionation obtained owes more to the size differences within each population of DNA molecules than to the known base compositional differences. The possibility of obtaining native, host DNA from infected cell material seems remote due to the probable breakdown of host genome by the infecting virus.

5.

(b) Caesium Chloride density gradient centrifugation.

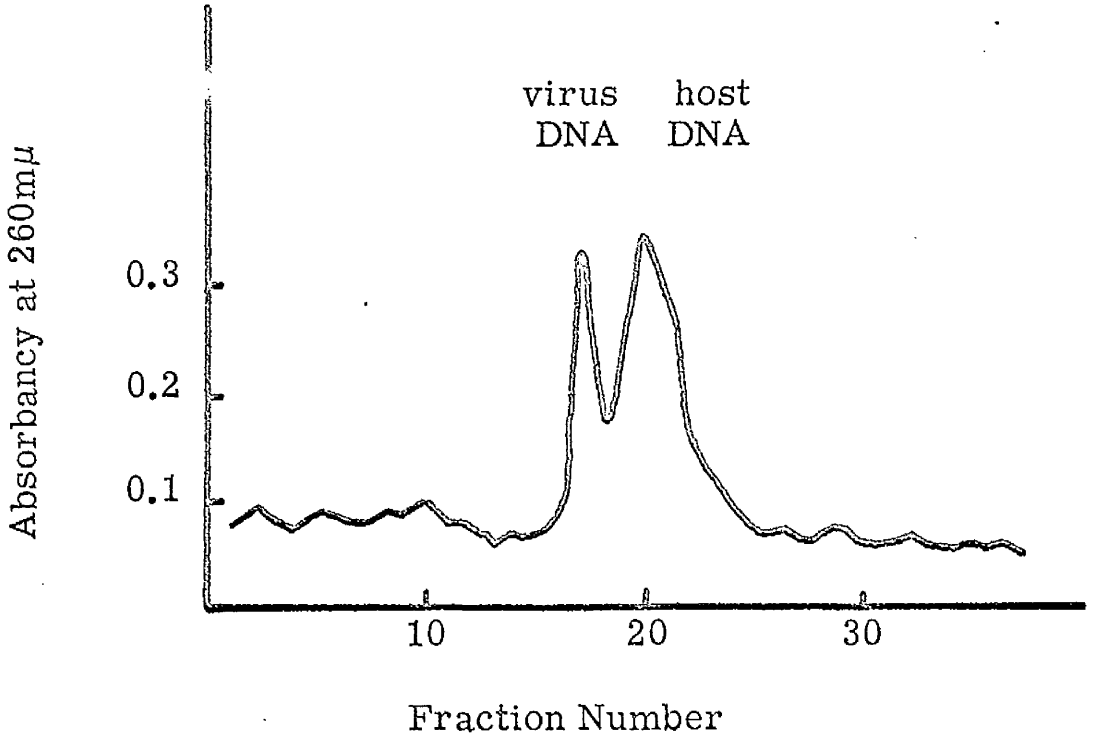
MAK columns did not effectively separate virus DNA from host DNA. However, centrifugation of the two DNAs to equilibrium in a gradient of CsCl should allow separation of these on the basis of their buoyant densities which will vary with the base compositions of the DNAs. Several times already this has been proved in analytical measurements using the Spinco Model E

F I G U R E 33.

Equilibrium centrifugation in CsCl of a mixture of Herpes Simplex virus and pHK21 (C13) DNAs.

The material was centrifuged at 25° in the SW39 rotor of the Spinco model L ultracentrifuge and collected in 0.1 ml. fractions.

FIGURE 33


virus DNA
host DNA
0.3
0.2
0.1
Absorbancy at 260mμ
10
20
30
Fraction Number

(see Figs. 22, 23).

A CsCl density gradient was set up for fractionation of host (BHK21 (C13)) and virus DNA in a mixture of the two. The material was centrifuged at 25° for 3 days in the Spinco Model L, and the gradient harvested in two-drop fractions collected from a puncture in the base of the tube. (Methods Sect. 8(b)). The CsCl content of every fourth fraction was estimated. (Methods Sect. 6(e)).

CsCl density gradient centrifugation on a preparative scale leads to an effective resolution of host and viral DNA (Fig. 33); however, as a method for obtaining sufficiently large quantities of pure viral DNA, this fractionation method is not suitable.

Pure Herpes Simplex virus DNA was therefore routinely prepared in bulk from highly purified virus particles exhaustively treated with DNase and RNase. (see Results Section 2).

RNA SYNTHESIS IN BHK21 (C13) CELLS BEFORE AND AFTER INFECTION WITH HERPES SIMPLEX VIRUS.

The Herpes Simplex virus particle contains no RNA (Russell & Crawford [191]), but in order to make protein specified by its DNA genome, the virus must be responsible for the synthesis at some time in the infective process of an RNA species, which functions as an intermediate in protein synthesis. The non-appearance of such RNA would contravene widely held views on the biochemical events during protein synthesis. It has been shown that virus-specific protein synthesis occurs in a cell after infection by Herpes virus (Russel et.al. [148]).

In this section of the work experiments on RNA metabolism in Herpes Simplex virus-infected cells will be described.

6. Fractionation of RNA.

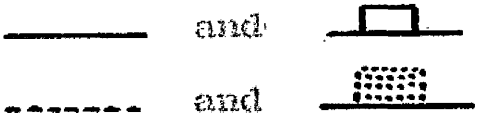
A necessary preliminary to the investigation of RNA metabolism is the definition of the most suitable method available for the fractionation of RNA.

Three such methods were examined and assessed for their usefulness. RNA was prepared from BHK21 (C13) cells (Keir & Wildy [362]), which had been labelled for 1 hour with [^{3}H] uridine, and was used as test

F I G U R E 34.

Chromatography of RNA from BHK21 (C13) cells on the MAK column.

Elution was achieved at 20° using an essentially linear gradient of 0.15M to 1.2M NaCl. A batch of 2M NaCl was employed following the gradient elution.



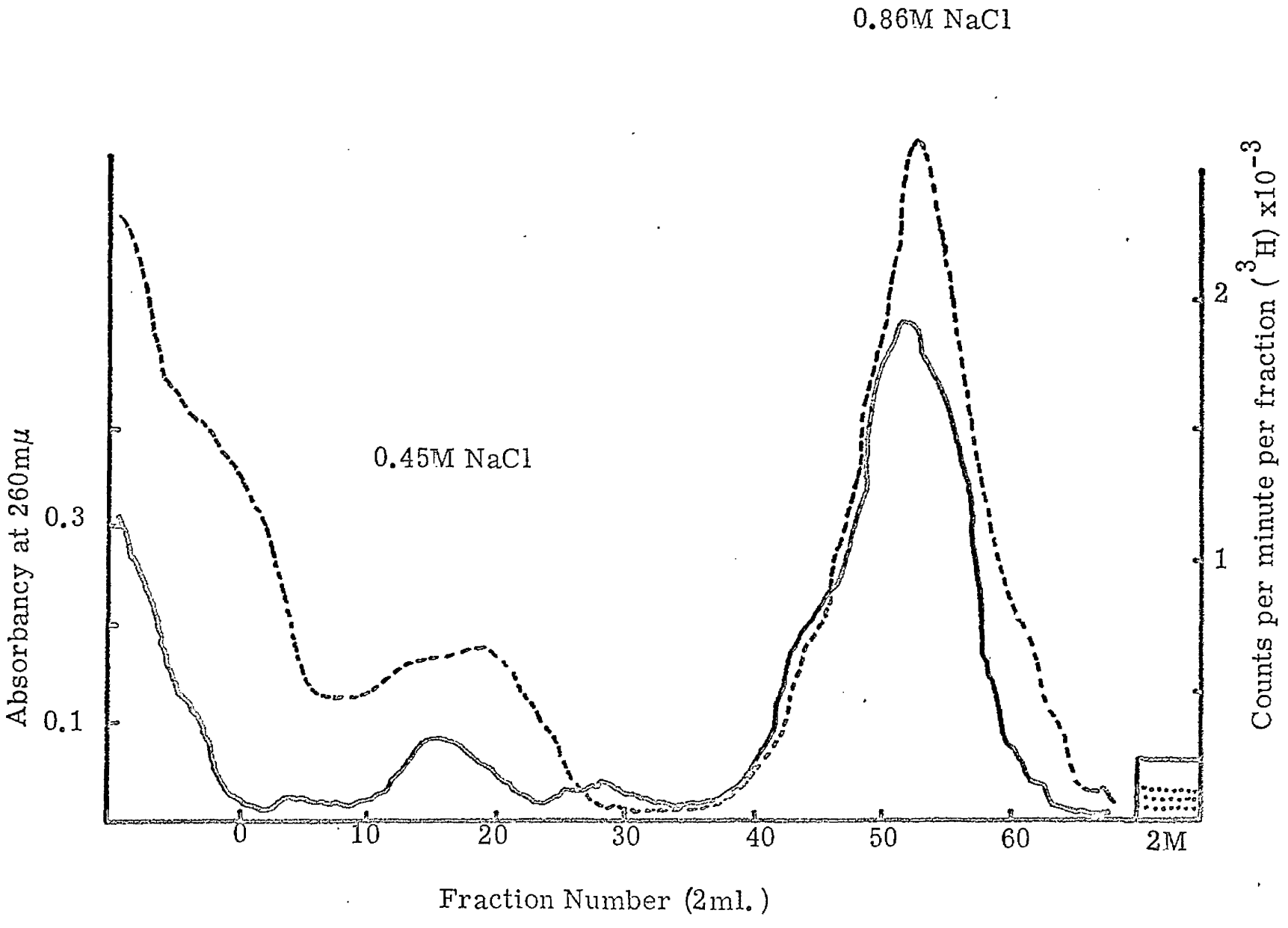

0.86M NaCl
0.45M NaCl
Absorbancy at 260mμ
0.3
0.1
0
10
20
30
40
50
60
2M
Fraction Number (2ml.)
Counts per minute per fraction (^{3}H) x10^{-3}
2
1

FIGURE 34

material in the following systems.

6.

(a) Chromatography on MAK columns.

400 ug. of the RNA was applied to a 10ml. MAK column and elution was achieved using a linear gradient of 0.15M to 1.2M NaCl in 0.05M sodium phosphate buffer pH 6.7. (Methods Sect. 2(b)).

The elution profile (Fig. 33) shows that the RNA has been resolved into three main components (a) material, certainly small and probably fragments of RNA, which has not been significantly retained by the methylated albumin (b) a peak at 0.45M NaCl and (c) a large peak at 0.86M NaCl. Components (b) and (c) correspond respectively to transfer RNA and ribosomal RNA in their elution properties under these conditions (Sueoka & Yamane [346]).

Each 2ml. fraction from the column was precipitated on to a "Millipore" membrane filter. Each disk was placed in a vial containing 10ml. of toluene-based scintillation fluid, and counted in the Nuclear Chicago liquid scintillation spectrometer (Methods Sect. 6(h)). Two major peaks of radioactivity (Fig. 34) correspond to peaks (b) and (c) of the absorbancy profile, and the specific activities are compatible with their identities

F I G U R E 35.

The composition of a sucrose gradient formed using the apparatus shown in Figure 20a and harvested in the device drawn in Figure 20b.

F I G U R E 36.

Sucrose gradient fractionation of RNA isolated from BHK21 (C13) cells.

The 5-20% sucrose gradient was centrifuged at 21,000 r.p.m. for 11.5 hr. in the SW39 rotor of the Spinco model L ultracentrifuge.

------ Radioactivity

_____ Absorbancy

FIGURE 35

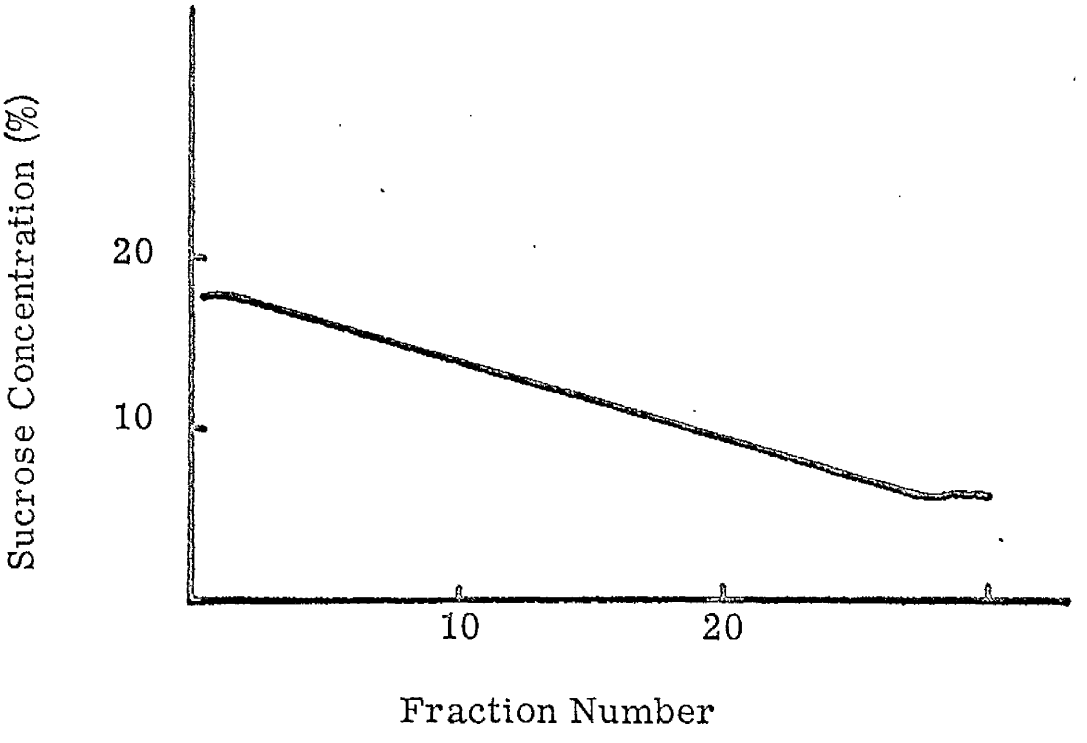
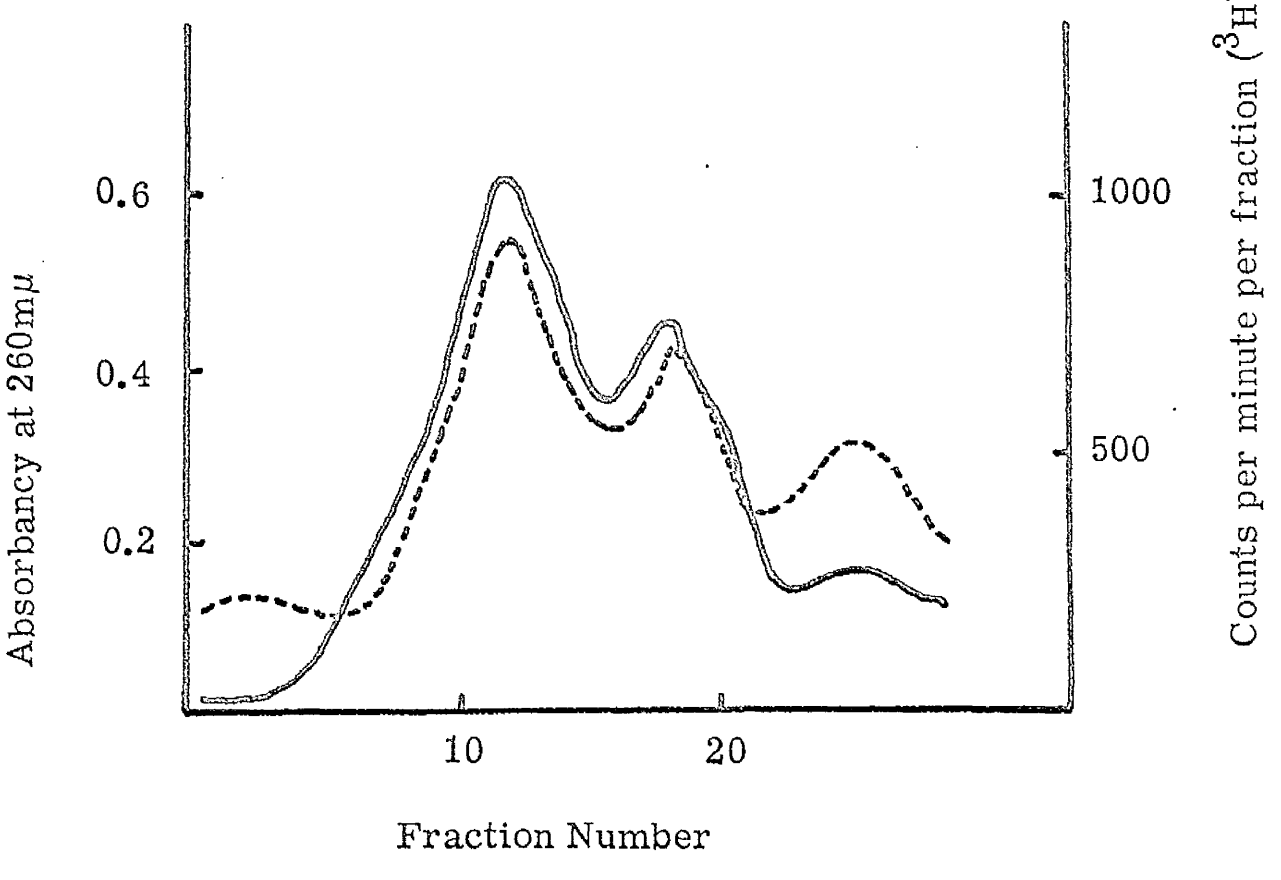


FIGURE 36



as mentioned above. In the region 0.15M to 0.40M NaCl, there is a broad distribution of radioactivity which is not well defined and which probably will be associated with the small molecular weight material in peak (a).

6.

(b) Sucrose Gradient Analysis.

The technique was developed by (Britten & Roberts [347]) and utilises the differing S values of RNA as a basis for fractionation; the gradient of sucrose stabilises for a short time the position of the molecules after centrifugation.

A sucrose gradient (5-20% w/v) was set up, centrifuged and harvested in the standard way (Methods Sect. 7(b)) to test its stability. Each fraction was measured for sucrose content using a Bellingham and Stanley sugar refractometer. The gradient remained essentially linear throughout the procedure, while a limited degree of mixing at both the top and bottom of the tube occurred; this was judged not to be critical (Fig. 35).

300 ug. of purified BHK21 (C13) cell RNA (see previous section) was fractionated in a 5-20% (w/v)

F I G U R E 37.

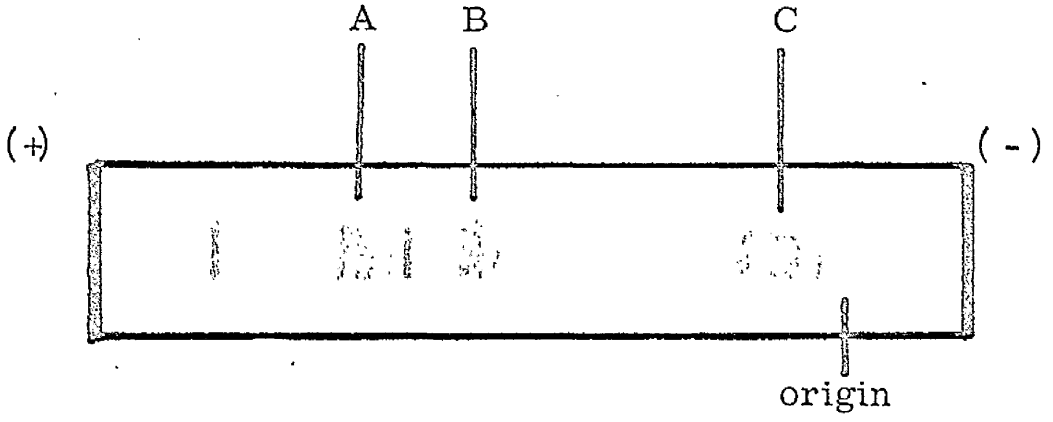
Agarose gel electrophoresis of RNA from BHK21 (C13) cells.

The major bands are as follows:

A,B - ribosomal RNA

C - sRNA

FIGURE 37



sucrose gradient, and each fraction measured for absorbancy at 260mμ in the Unicam SP 500 spectrophotometer. (Fig. 36). Three peaks, corresponding to the two ribosomal RNA species (peaks I and II) and transfer RNA (peak III) were resolved under these conditions. Each fraction was assayed for radioactivity in 8ml. of dioxane-based scintillator and counted in the Nuclear Chicago Liquid Scintillation Spectrometer (Methods Sect. 6(h)(i)). The radioactivity levels are coincident with absorbancies, apart from the peak III region, whose absorbancy distribution is broad.

6.

(c) Electrophoresis on Agarose Gel.

50ug. of the above RNA was subjected to electrophoresis through agarose gel (Methods Sect. 7(c). Several bands can be seen (Fig. 37), the largest being ribosomal (A&B) and transfer (C) RNA's respectively (McIndoe [348]). The RNA, although labelled with [^{3}H] uridine, could not conveniently be counted or recovered from the gel, so that no comparison of radioactivity and absorbancy was undertaken.

7.

7. ASSESSMENT OF METHODS OF RNA FRACTIONATION.

If we assume that agarose electrophoresis does not break down the RNA applied to it, the technique is capable of much greater resolution of RNA components than are the other methods used. However, in its present form, it is useful only analytically, and was therefore too limited in scope for the purpose of the present project. Fractionation on MAK columns is convenient, but involves extensive dilution of material in solutions of high salt concentration. This is not favourable to the measurement of radioactivity: moreover, it does not separate ribosomal RNA into its component species, and this lack of specific resolution is not desirable.

Sucrose gradient analysis was therefore chosen because it is rapid, convenient and resolves species of RNA to an acceptable extent.

8. ISOLATION OF RNA.

(a) A comparison of the methods of Kirby and of Eason, Cline and Smellie.

Two preparative methods for isolation of RNA were investigated prior to use: the method favoured would be rapid, convenient and capable of extracting all forms of cellular RNA in one operation.

TABLE 7.

Comparison of RNA prepared from BHK21 (C13) cells by the method of Kirby [343] and by the method of Eason *et.al.* [343].

The cells had been exposed to radioactive precursor for 30 minutes prior to harvesting.

TABLE 2

Method of Extraction	Extract Absorbancy at		Interface Absorbancy at		Percentage Radioactivity at Interface
	260mμ	280mμ	260mμ	280mμ	
Kirby	2.34	1.31	1.21	1.96	17
Mason, Cline & Smillie	2.52	1.32	not measured		9

RNA was extracted from BHK21 (C13) cells (which had been exposed to [^{3}H] uridine for 30 minutes.) either by the method of Kirby (Methods Sect. 5(a)) or by the Eason, Cline and Smellie technique (Methods Sect. 5(b)). Table 7 gives details of the yield of RNA, the protein content of the RNA and the efficiency of extraction of the RNA using these procedures. The yield of RNA does not differ between the methods, but more RNA remains behind in the "interphase" using the Kirby method. Also the "Kirby RNA" seems, on the basis of the A260/A280 ratio, to be contaminated to a greater extent with protein. Moreover, the Eason, Cline and Smellie method is the more rapid one, and was accordingly used for all subsequent preparations of RNA.

8.

(b) Examination of Factors affecting the isolation of RNA.

Several factors influence the composition of an RNA fraction obtained from infected and uninfected cells in a pulse-label type of experiment. The first of these factors relates to the method of pulse-labelling cell samples and harvesting them prior to isolation of RNA.

Eight 80 ounce bottles of BHK21 (C13) cells (each 2×10^{8} cells) were treated in batches of two in one of

the following four ways. (i) Cells were removed from the bottles using Trypsin and EDTA (Methods Sect. 1(b)) suspended in Eagle's medium containing 10% Tryptose phosphate and 10% Calf serum (Methods Sect. 1(a)) (ETC) at 3 x 10^7 cells/ml., and shaken at 37° for 30 mins. [^{3}H] uridine (2μC/ml) was added to the medium and, after a further 30 minutes, the cells were harvested by centrifugation, washed in ice-cold phosphate-buffered saline and stored in 0.1% SDS, 0.2% bentonite, and 0.01M sodium acetate buffer pH 5.2 at -70°C. (ii) Treatment was as described above except that shaking was continued for 3.5 hours before the 30 minute pulse and harvesting. (iii) The medium was removed from the 80 oz. bottles and replaced with fresh ETC containing [^{3}H] uridine at 2μC/ml. After 30 minutes the medium was removed and Ballotini beads (size 12) and SDS and bentonite to final concentrations 0.1% and 0.2% in 0.01M sodium acetate buffer pH 5.2 were added to the culture. This treatment removed the cells from the glass and the beads were removed by centrifugation before storing the cells at -70°. (iv) Bottles were treated as in (iii) but harvesting of the cells was carried out using the Trypsin and EDTA procedure outlined for (i) and (ii). Washed cells were suspended in 0.1% SDS and 0.2% bentonite as

before.

RNA was extracted undialysed from all four samples using the Eason, Cline and Smellie method (Methods Sect. 5(b)) and analysed on sucrose gradients (Methods Sect. 7(b)). Absorbancy at 260mμ and radioactivity in each fraction were determined as before (Results Sect. 6(b)). Examination of the sedimentation profiles (Fig. 38) of all four RNA samples shows that treatment (iii) to some extent and treatment (iv) to a large extent promoted breakdown of the RNA. On the other hand samples (i) and (ii) provide RNA whose absorbancy pattern does not exhibit extensive degradation of RNA. Sample (ii) has a larger 4S peak than (i) and this may be due to a small amount of breakdown arising from the shaking period of 3.5hours. To some extent this explanation is substantiated by the radioactivity pattern, which shows more larger material present in (i) than in (ii). Since neither preparation was dialysed, some free $[^3H]$ uridine and derivatives are to be expected at the top of each gradient. We can draw the conclusion, then, that method (i) is a satisfactory one if the cells are not shaken for too long a period.

Dialysis of the isolated RNA was not carried out

F I G U R E 38.

Sucrose gradient fractionation of RNA isolated from uninfected BHK21 (C13) cells using several extraction procedures.

(i) Cells harvested in the standard way.

(ii) As (i) after 3.5 hr. shaking.

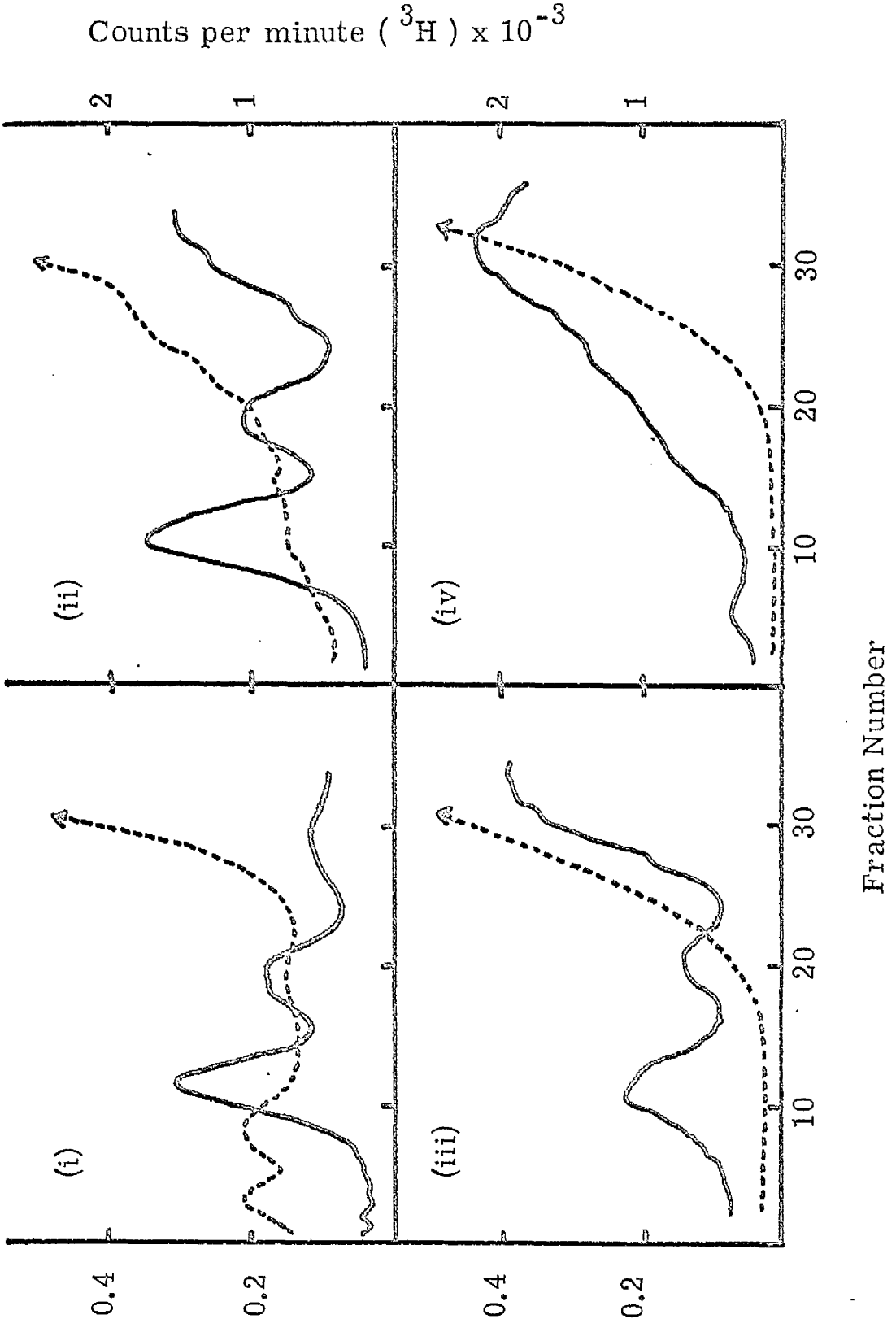
(iii) Pulsed *in situ* and removed with SDS.

(iv) As (iii) but harvested as (i).

——— Absorbancy at 260mu

- - - - - Radioactivity

FIGURE 38


Counts per minute (^{3}H) x 10^{-3}
2
1
(ii)
(iv)
(i)
(iii)
0.4
0.2
10
20
30
Fraction Number
Absorbancy at 260mμ

F I G U R E 39.

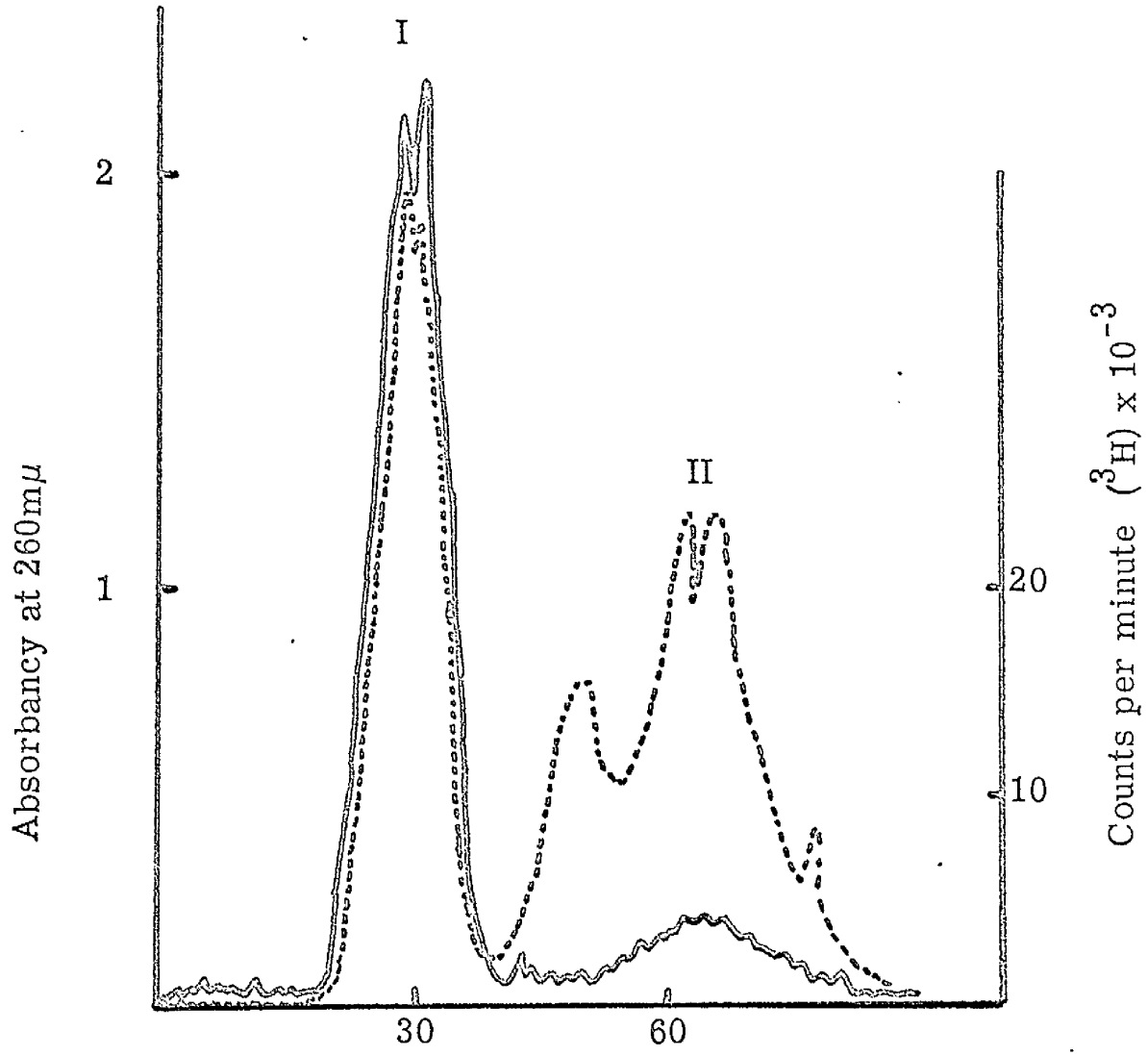
Gel filtration of radioactive RNA from BHK21 (C13) cells.

The RNA was prepared from cells exposed to radioactive uridine for 30 minutes before harvesting. The gel employed was Sephadex G25.

—————— Absorbancy at 260mμ

– – – – – – Radioactivity

FIGURE 39


I
II
2
1
20
10
30
60
Absorbancy at 260mμ
Counts per minute (^{3}H) x 10^{-3}
Fraction Number

since the procedure is time-consuming and may also lead to breakdown of particularly labile RNA fractions. It was hoped that the precipitation and washing steps which form part of the isolation method would be sufficient to remove small contaminating material from the RNA preparation. This, however, was not the case (Fig. 38) and the following method was investigated in this connection.

8.

(c) Gel Filtration of Isolated RNA.

500ug. of RNA from 30 min. pulse-labelled BHK21 (C13) cells prepared using method (i) above was applied to a column of Sephadex G.25, 10cm. x 1cm. (Methods Sect. 2(c)). The RNA was washed from the column using 0.01M sodium acetate buffer pH 5.2 containing 0.05M NaCl and 10^{-3}M $MgCl_2$, and collected in 0.1ml. fractions. The elution pattern is shown in Fig. 39 where the absorbancy at 260mμ is distributed between two peaks, I and II. Measurement of radioactivity using the method detailed in Methods Sect. 6(h)(i) gives peaks coincident with absorbancy peaks I and II and a third peak between these two. However, peak I represents material which is excluded by the gel

F I G U R E 40.

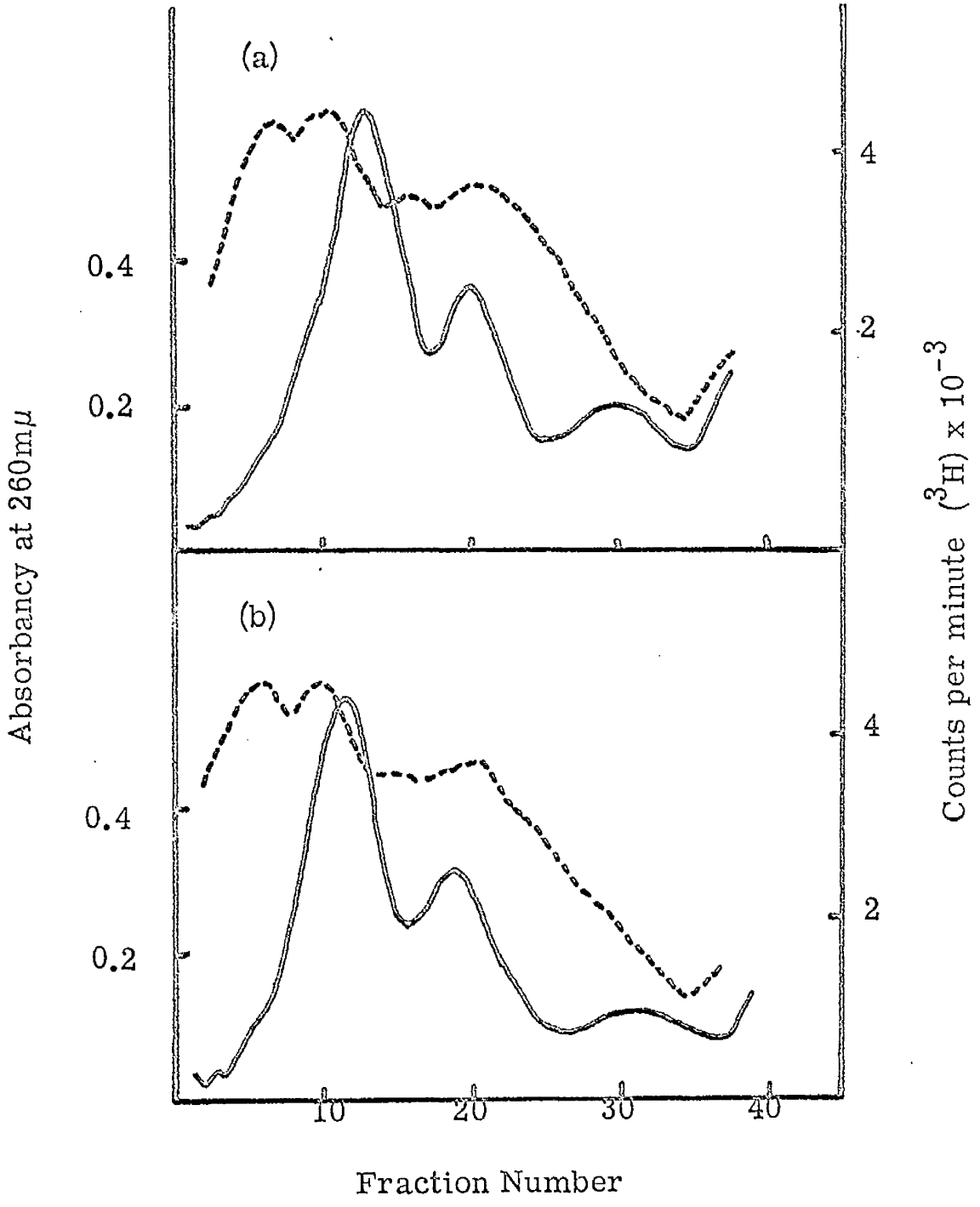
Analysis of RNA on sucrose gradients after gel filtration.

The RNA was prepared from peak I shown in Figure 39, and is derived from BHK21 (c13) cells. (Figure 40a). Figure 40b is identical to the above, but was exhaustively treated with DNase prior to gel filtration.

———— Absorbancy at 260mμ

– – – – – – Radioactivity

FIGURE 40


(a)
(b)
0.4
0.2
4
2
10
20
30
40
Absorbancy at 260mμ
Counts per minute (^{3}H) x 10^{-3}
Fraction Number

and therefore has a nominal m.w. above 5×10^3. RNA (or oligonucleotide) of smaller size is not of interest and represents breakdown and contamination with free [^{3}H] uridine and uridine derivatives.

8.

(d) Analysis of Sephadex-treated RNA on Sucrose gradients.

The RNA from Peak I (Fig. 39) was collected and layered onto a linear (5 - 20% w/v) gradient of sucrose. The sedimentation pattern (Methods Sect. 7(b)) (Fig. 40a) clearly shows the two peaks of ribosomal RNA and the peak of transfer RNA, the size of the latter peak suggesting little or no contamination with small material. The radioactivity gives a wide distribution of rapidly-labelled RNA. This spread of label encompasses ribosomal and transfer RNA as well as material not associated with significant absorbancy and represents satisfactory fractionation. Fig. 40b shows the result of an analysis of RNA identical to the above, except that this material was exhaustively treated with DNase at twice the standard level (Methods Sect. 5(b)) before passage through Sephadex G.25, which clearly is effective in the removal of contamination from RNA. It was suspected that perhaps DNase action on these RNA

F I G U R E 41.

Sucrose gradient analysis of RNA from BHK21 (C13) cells prepared using several methods of RNase inhibition.

(a) Using Bentonite and fresh phenol.

(b) Using Bentonite.

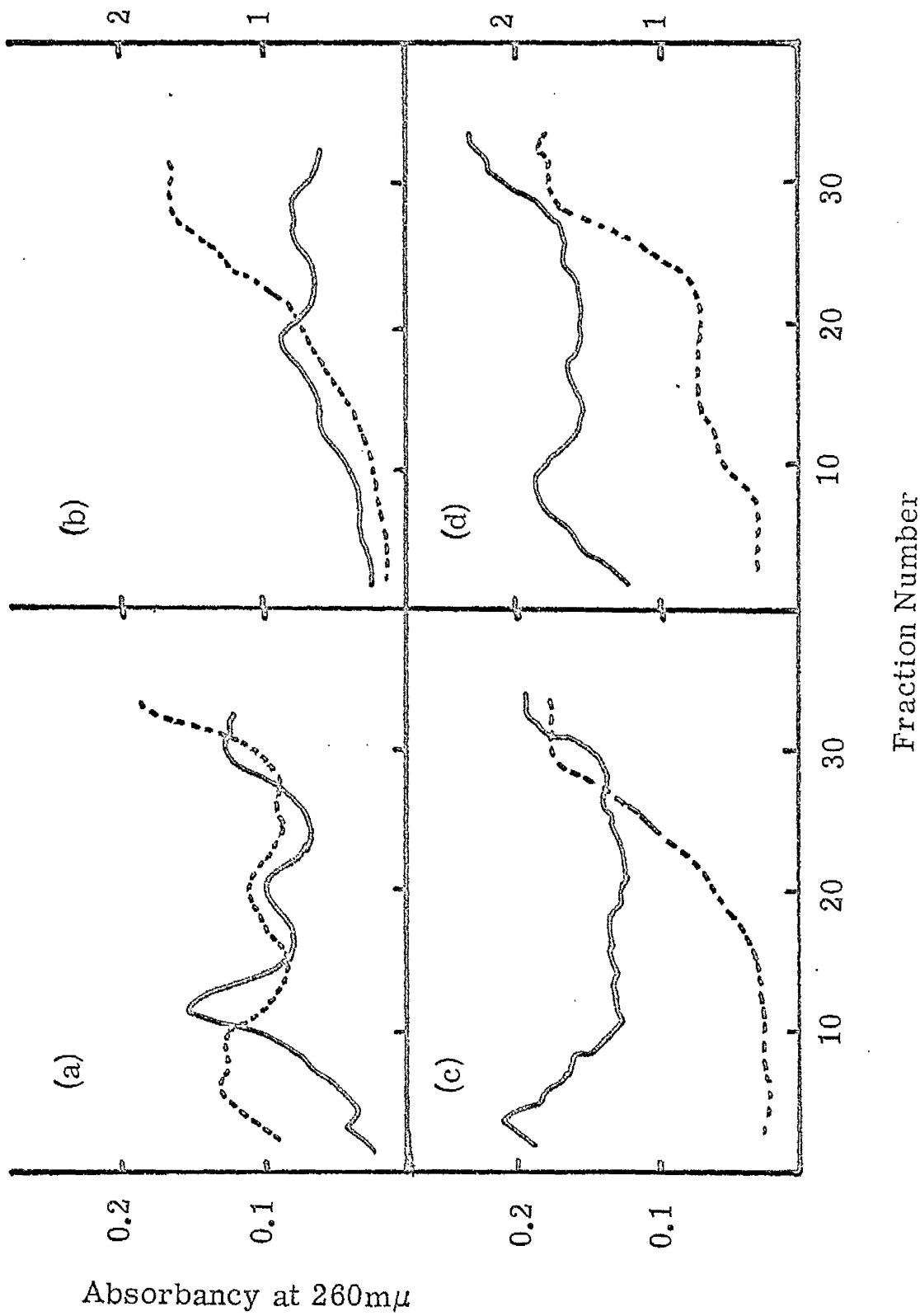
(c) Using fresh phenol.

(d) Using fresh phenol and 8-OH quinoline.

——— Absorbancy at 260mμ

- - - - - - Radioactivity

FIGURE 41


Counts per minute (^{3}H) x 10^{-3}
2
1
(b)
(d)
(a)
(c)
0.2
0.1
10
20
30
Fraction Number
Absorbancy at 260mμ

preparations was not thorough enough, but the fact that Figs. 40a and 40b are superimposable clearly disposes of this criticism.

8.

(c) Assessment of Methods of Inhibition of RNase Activity during RNA Isolation.

Four 80 oz. bottles of BHK21 (C13) cells were pulse-labelled and harvested separately as described in Results Sect. 8(c). The cells from bottle b were treated for RNA isolation by the Eason, Cline and Smellie method (Methods Sect. 5(b)). Bentonite (Methods Sect. 4) was used in this case, but the phenol employed had not been freshly redistilled. RNA was extracted from bottle a by the above technique using freshly redistilled phenol. Bottles c and d were extracted for RNA using redistilled phenol and without bentonite (bottle c) or with 0.1% 8-OH Quinoline in the phenol (bottle d). This last compound is employed by Kirby (Methods Sect. 5(a)), probably as an RNase inhibitor.

All four RNA's were analysed on a linear gradient (5-20% w/v) of sucrose after passage through Sephadex G.25; the absorbancy and radioactivity patterns are shown in Fig. 41.

This figure shows clearly that the use of phenol which has not been redistilled produces degraded RNA: it shows that the presence of bentonite during the extraction process is essential and that 8-OH Quinoline is not an effective inhibitor of RNA breakdown under these conditions. RNA degradation in this system is probably due to the action of RNase released from the disrupted cell: this is borne out by the action of bentonite, a known inhibitor of RNase (Singer & Frankael-Conrat [363]).

8.

(f) Treatment of Sucrose with Bentonite.

The possibility that commercial "Analar" sucrose contains RNase has been discussed by Sanders (Sanders [349]). Accordingly the RNA sample analysed in Fig. 40a was refractionated on an identical gradient of sucrose treated with Bentonite in the standard way (Methods Sect. 7(a), 7(b)).

The result obtained was identical with that in Fig. 40a. Nevertheless, Bentonite-treated sucrose was routinely employed in the event that a batch of sucrose might contain RNase activity.

F I G U R E 42.

Fractionation of Krebs II Ascites cell and BHK21 (C13) cell RNA on sucrose gradients.

(a) Krebs II ascites cell RNA.

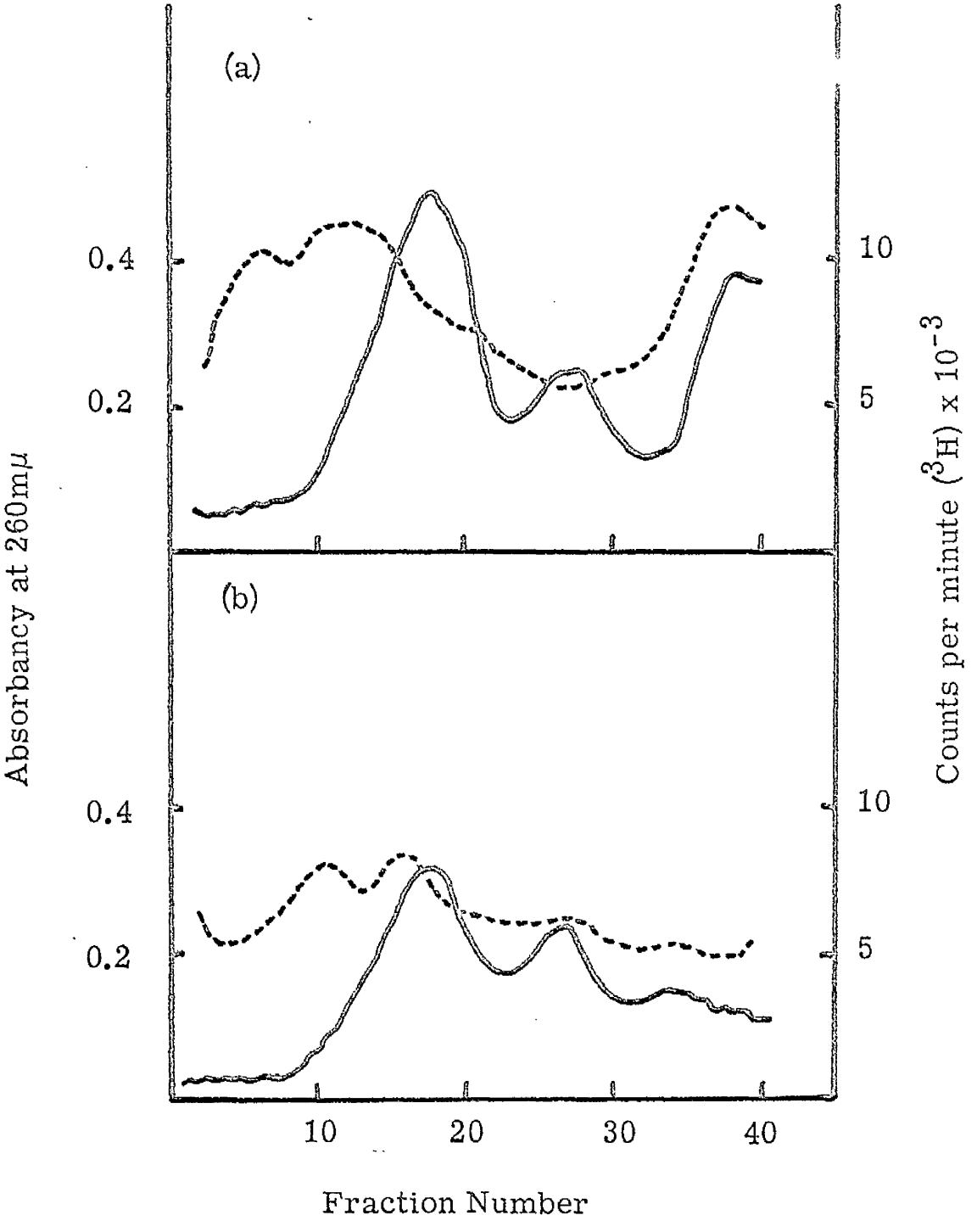
(b) BHK21 (C13) cell RNA.

Both RNA samples were exposed to radioactive uridine for 30 minutes before isolation of RNA.

——— Absorbancy at 260mμ

- - - - - - - Radioactivity

FIGURE 42


(a)
(b)
0.4
0.2
10
5
10
20
30
40
Absorbancy at 260mμ
Counts per minute (^{3}H) x 10^{-3}
Fraction Number

8.

(c) Test fractionation of Krebs II Ascites cell RNA and the use of Dialysis.

In order that the method to be used routinely for the isolation of RNA from BHK21 (C13) cells could be tested in a system whose RNA had previously been characterised, Krebs II ascites-tumour cells were pulse-labelled with [^{3}H] uridine for 30 minutes prior to extraction of RNA by the standard method (Methods Sect. 5(b)). Concomitantly, BHK21 (C13) cells were treated identically. Both RNA preparations were dialysed against one change (2L.) of 0.01M sodium acetate buffer, pH5.2 containing 10^{-3}% bentonite, 0.05M NaCl and 0.001M $MgCl_2$ prior to analysis on linear gradients of sucrose: the dialysis, which was carried out in EDTA-washed tubing replaced gel filtration as a means of removing small molecular material from the RNA sample. Comparison of Fig. 42a with a published analysis of Krebs II ascites cell RNA (Eason et.al. [343]) reveals little difference between the two preparations, both on the basis of absorbancy at 260mμ and radioactivity. Fig. 42b is similar to earlier analyses of pulse-labelled BHK21 RNA (cf. Fig. 40a); from these fractionation experiments on undegraded RNA of BHK21 (C13) cells a characteristic pattern of incorporation emerges.

The salient features are (i) RNA of one or two species larger than the heavier ribosomal RNA (ii) labelling of ribosomal RNA (iii) synthesis of 4S RNA. Dialysis under the above conditions seemed to have no deleterious effect on the preparation and was adopted into the standard preparative procedure, since, although gel filtration is conveniently carried out on a small number of samples, dialysis of a large number is easier.

After the sucrose gradient analysis carried out in this experiment, the centrifuge tube was carefully dried and the small pellet formed during centrifugation was dissolved in 0.1M KOH, in order to extract any RNA which may have sedimented. This KOH extract (which had no absorbancy at 260mμ) was assayed for radioactivity and was found to contain less than 0.5% of the total material applied to the gradient: however, such material was consistently observed in future sucrose gradient analyses.

9. RNA SYNTHESIS AFTER HERPES INFECTION.

A satisfactory method of pulse-labelling, storage, extraction and fractionation of RNA from cells under conditions analogous to those needed when using virus-infected cells, having now been worked out, a series of experiments with infected and uninfected cells

was initiated.

(a) Effect on RNA synthesis of Infection of BHK21 (C13) cells with Herpes Simplex virus.

Four 80 oz. bottles of BHK21 (C13) cells were harvested using Trypsin and EDTA (Methods Sect. 1(b)) and the pooled cultures split into two equal portions of 2×10^8 cells each. Both samples were suspended in ETC at 5×10^6 cells/ml, and one batch infected with Herpes Simplex virus at a multiplicity of exposure of 15 PFU/cell. All cells were shaken for 20 minutes at 37°. Portions (2.5×10^7 cells) were dispersed into flat medicine bottles (20 oz.) and incubated at 37°. Immediately, four bottles (10^8 cells) of uninfected BHK21 (C13) cells were exposed to 4µC/ml. of $[^3H]$ uridine. After 30 minutes growth, the medium was poured off and the cells were scraped from the glass and washed with phosphate-buffered saline at 4°. The cell pellet was taken up in SDS, bentonite and sodium acetate buffer as described previously (Results Sect. 8(b) (i)). Similar 30 minute pulses of $[^3H]$ uridine were carried out in uninfected cells from 3 to 3.5 hours and in infected cells from 1 to 1.5 hours and 3 to 3.5 hours after infection. Time 0 corresponds to the completion of the 30 minute virus adsorption period. RNA was isolated

F I G U R E 43.

The effect on RNA synthesis of infection of BHK21 (C13) cells with Herpes Simplex virus.

All samples were exposed to radioactive uridine for 30 minutes before harvesting, and the RNA analysed on sucrose gradients.

(a) Uninfected, pulsed from 0 - 30 minutes after infectio

(b) Uninfected, pulsed from 3 - 3.5 hr.

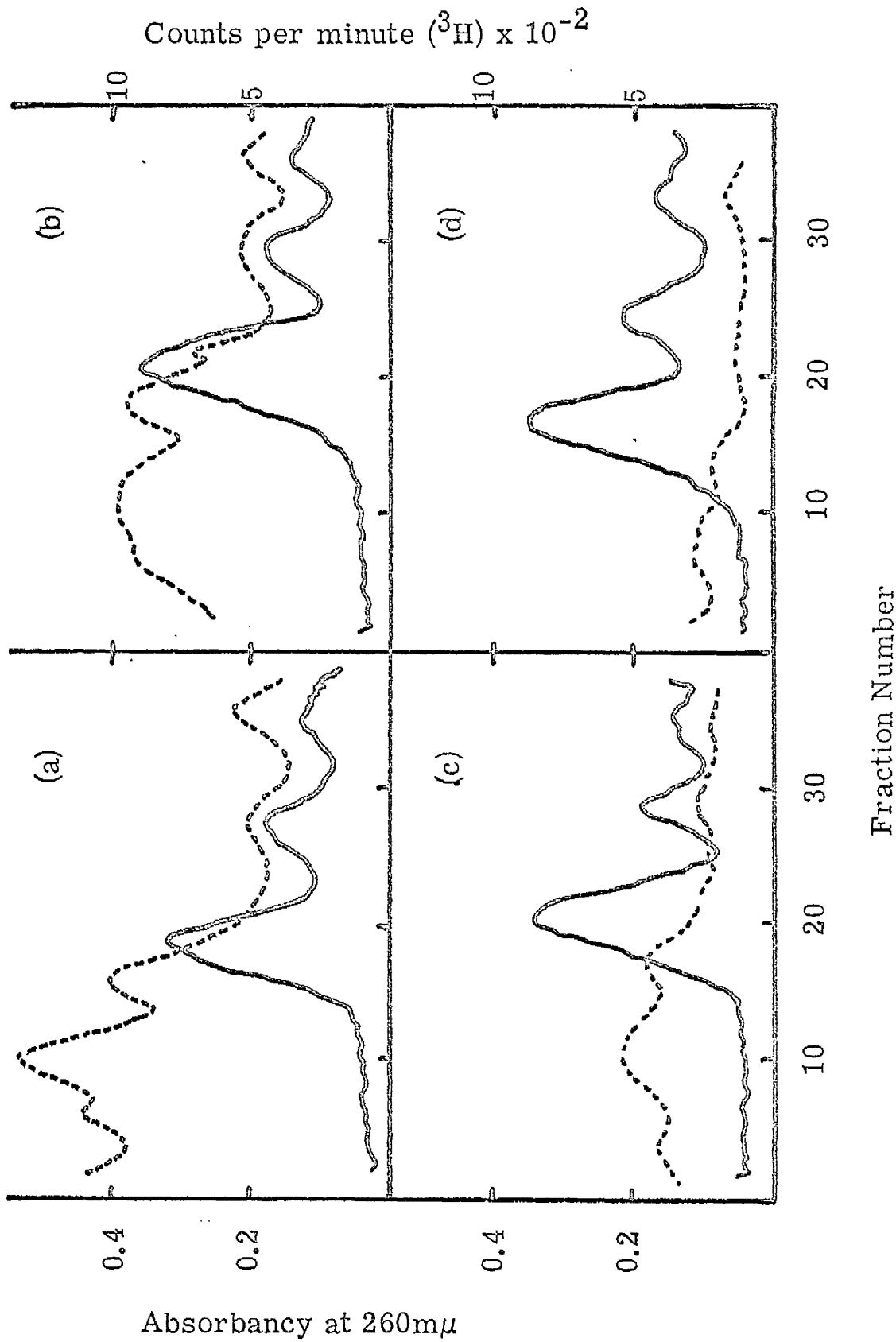
(c) Infected, pulsed from 1 - 1.5 hr.

(d) Infected, pulsed from 3 - 3.5 hr.

——— Absorbancy at 260 mμ

- - - - - - - Radioactivity

FIGURE 43


Counts per minute (^{3}H) x 10^{-2}
10
5
(b)
(d)
(a)
(c)
0.4
0.2
10
20
30
Absorbancy at 260mμ
Fraction Number

from all four samples and analysed on sucrose gradients (Methods Sect. 7(b)). The control (uninfected) pictures (Fig. 43. a,b) are similar and verify that the conditions of infection and pulse-labelling do not alter the RNA complement of the uninfected cells; the characteristic rapidly-labelled RNA profile is evident in both analyses (cf. Fig. 42a). Figs. 43c and d show a marked decrease in uptake of [^{3}H] uridine relative to the uninfected cultures, Fig. 43d (3 to 3.5 hours after infection) to a larger extent than Fig. 43c (1 to 1.5 hours after infection). However, this quantitative change in RNA synthesis induced after infection is not, (at least at the investigated time intervals) accompanied by a qualitative change in the pattern of incorporation, i.e., all species of RNA visible in uninfected analyses are present in infected samples, but to a smaller degree. If all RNA synthesised in BHK21 (C13) cells is regarded as being under the control of the cell DNA, then the conclusion may be drawn that such host-controlled synthesis is diminished as a result of infection.

9.

(b) <u>Effect of Mitomycin C on synthesis of RNA.</u>

No qualitative change in the pattern of synthesis

of BHK21 (C13) cell RNA was observed in the last experiment following infection with Herpes Simplex virus; the reason for this may be that residual cell-directed incorporation is masking virus-directed RNA synthesis. In an attempt to overcome this difficulty, cells were treated with Mitomycin C prior to infection with Herpes Simplex virus. Mitomycin C acts on the DNA of an organism and inhibits primarily DNA synthesis, probably by cross-linking the DNA strands (Iyer & Szybalski [285]). A secondary effect of Mitomycin C, especially at higher concentrations, is inhibition of RNA synthesis. Since Mitomycin C binds to DNA, its effect in the living cell may be retained by allowing binding to take place and then washing away the excess unbound material.

Accordingly, 4×10^8 cells were exposed to 10µg./ml. Mitomycin C for 4 hours, and the excess Mitomycin C was removed by washing the cell sheet twice with ETC. Two hours later the cells were harvested (Methods Sect. 1(b)) and split into two portions of 2×10^8 cells. Both cell batches were shaken at 37^o for 20 minutes, one in the presence of Herpes Simplex virus at a multiplicity of exposure of 20 PFU/cell, and both were then plated into 20 oz. medicine bottles at 2.5×10^7 cells/bottle. Two identical cell samples, one infected and one uninfected,

F I G U R E 44.

The effect of Mitomycin C on the synthesis of RNA before and after infection with Herpes Simplex virus.

All samples were exposed to radioactive uridine for 30 minutes before harvesting and the isolated RNA fractionated on sucrose gradients

(a) Uninfected

(b) Uninfected plus Mitomycin C

(c) Infected

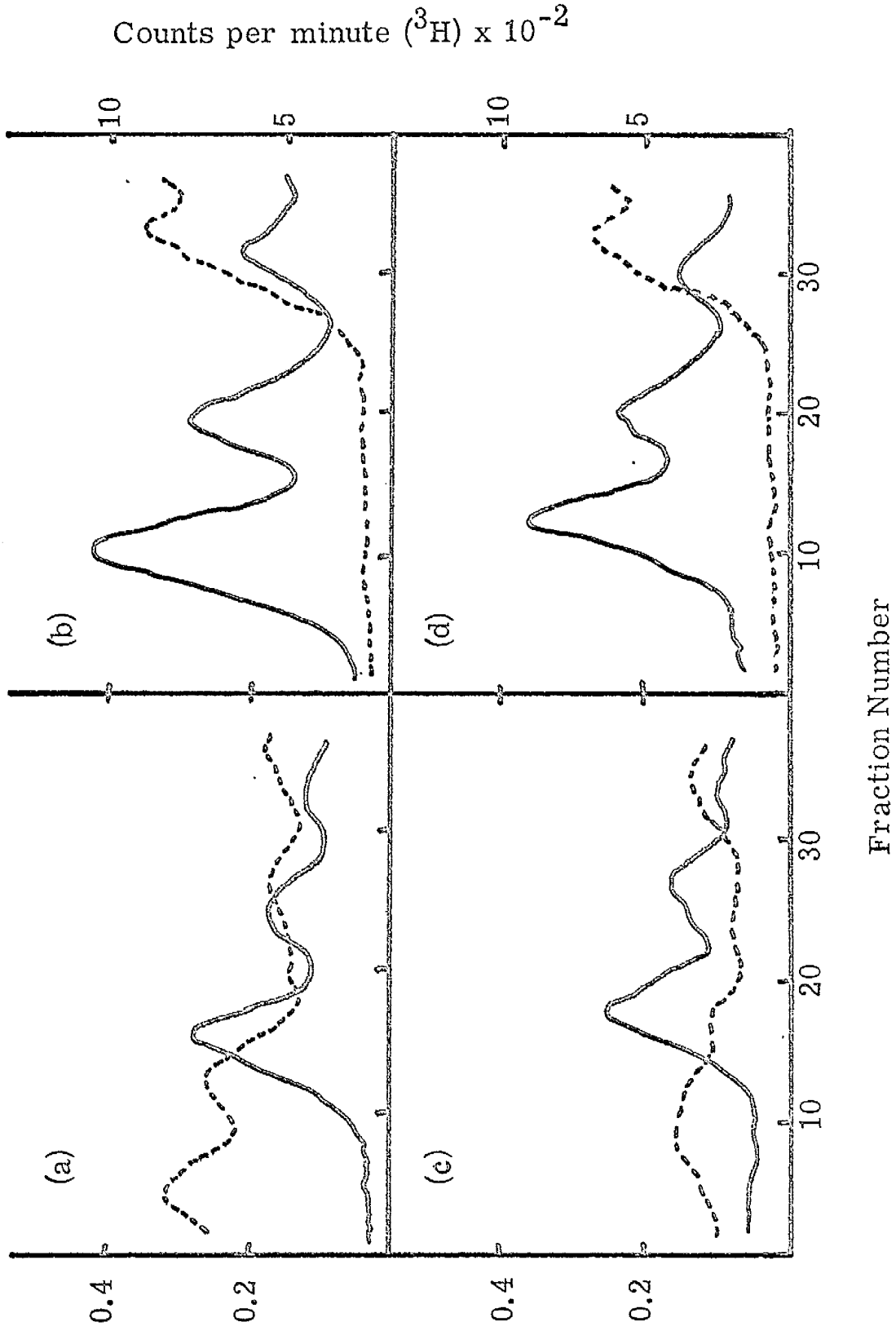
(d) Infected plus Mitomycin C.

The four samples were pulsed from 3 to 3.5 hr. after infection.

——— Absorbancy at 260mμ

– – – – Radioactivity

FIGURE 44


Counts per minute (^{3}H) x 10^{-2}
10
5
(b)
(d)
(a)
(c)
0.4
0.2
1
10
20
30
Fraction Number
Absorbancy at 260mμ

were treated in the above way, but no Mitomycin C was used in either sample. Growth was continued at 37° in all samples using 50 ml. of ETC, and, between 3 and 3.5 hours after infection, all four were pulsed with [^{3}H] uridine (4μC./ml.). The cells were harvested and RNA was prepared and analysed on sucrose gradients using the standard procedure (Results Sect. 9a). In none of the four analyses (Fig. 44) has the presence of Mitomycin C or Herpes Simplex virus altered the normal RNA absorbancy pattern (cf. Fig. 43a), but it is also evident that Mitomycin C has not allowed RNA synthesis (except some 4S material) to take place in control or virus-infected cells at this time.

10. PREPARATION OF RNA LABELLED WITH ^{32}P.

Over a 30 minute pulse period, rapidly-labelled RNA in BHK21 (C13) cells conforms to a characterisable pattern (cf. Fig. 43a). The quantitative alteration in this pattern revealed after infection with Herpes Simplex virus (Fig. 43) may reflect a replacement of host-specified RNA production with virus-coded material. It was decided to examine this possibility with reference to the labelled RNA fractions with an S-value higher than that of the heavier ribosomal species, since it should be

TABLE 8.

Distribution of ^{32}P among the nucleotides of RNA from Herpes Simplex virus-infected and uninfected BHK21 (C13) cells.

TABLE 8

Sample	% of total $^{32}_{in}P$ incorporation				GMP + CMP(%)
	GMP	AMP	CMP	UMP	
BHK 21 45S RNA	34.7	18.5	34.1	12.7	68.8
BHK 21 28S RNA	39.6	18.2	27.5	14.7	67.1
45S RNA (infected)	30.2	17.2	34.0	18.6	64
28S RNA (infected)	37.8	19.1	26.2	16.8	64

F I G U R E 45.

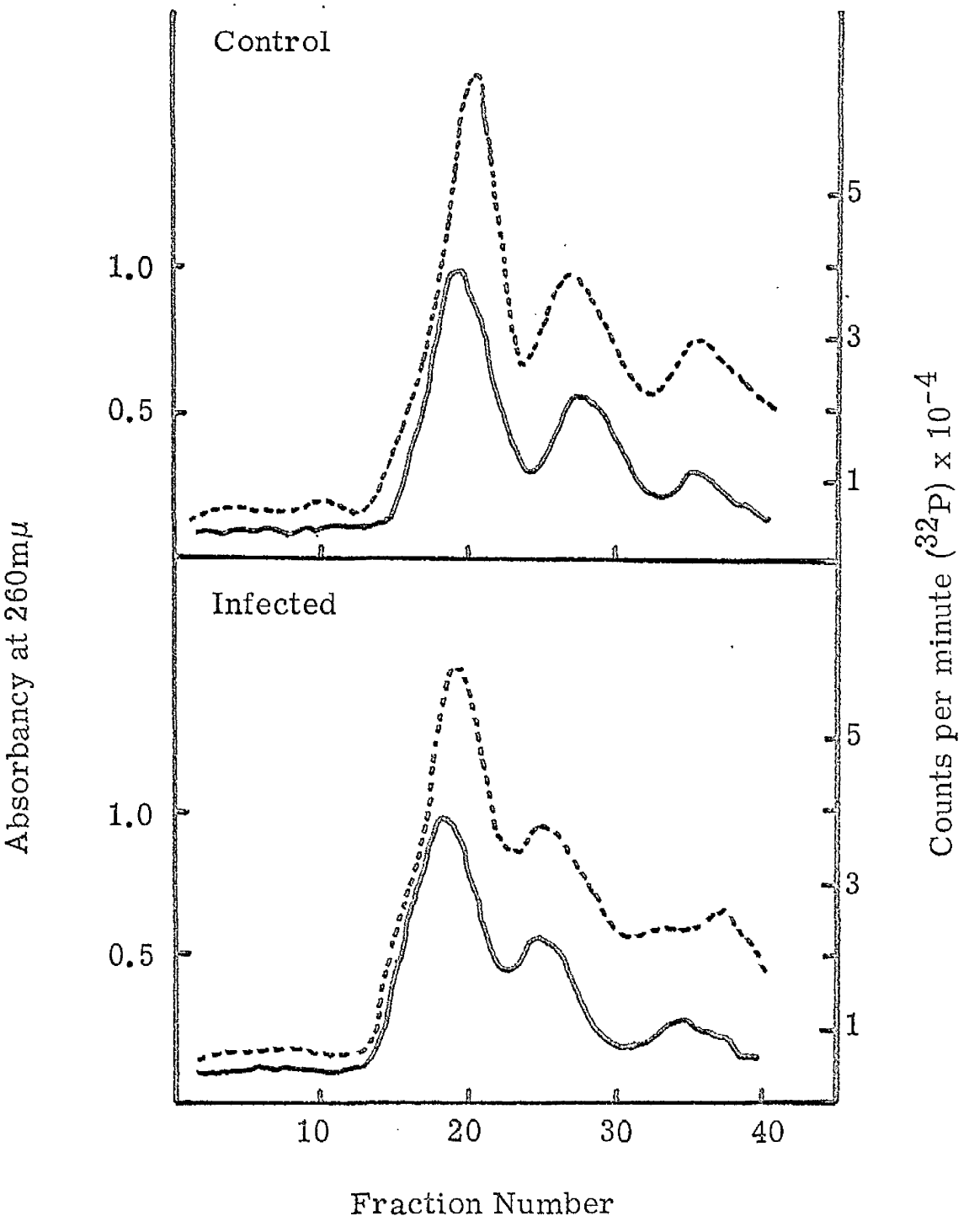
Fractionation of RNA labelled with ^{32}P from BHK21 (C13) cells before and after infection with Herpes Simplex virus.

The labelled RNAs, isolated 3 hr. after infection, were analysed on sucrose gradients. '45S' RNA was subsequently isolated from fractions 1 to 6, and '28S' RNA from fractions 13 to 19 in both analyses.

——— Absorbancy at 260mμ

– – – – – Radioactivity

FIGURE 45


Control
Infected
1.0
0.5
5
3
1
10
20
30
40
Absorbancy at 260mμ
Counts per minute (^{32}P) x 10^{-4}
Fraction Number

possible to obtain this fraction uncontaminated with other types of RNA. Since the difference between host and virus DNA base composition is so great (41% and 68% G plus C respectively), RNA derived from these two DNAs should be recognisable on the basis of their base compositions.

Two bottles of BHK21 (C13) cells were equilibrated with ^{32}P-orthophosphate (2mC./10^8 cells) for 24 hours. The medium was then removed and one half of the cells was infected with Herpes Simplex virus in the usual way (Methods Sect. 1(a)). After 3 hours infection the two samples were harvested and RNA was extracted from them by the standard method (Methods Sect. 5(b)). This RNA was fractionated on sucrose gradients and one fifth of each fraction was analysed for absorbancy and radioactivity (Fig. 45)). The remaining four-fifths of the relevant fractions (Fig. 45) were pooled, co-precipitated with 400µg. of yeast RNA and analysed (Methods Sect. 7 (d), (e), (f)) to find the distribution of ^{32}P among the nucleotides of the synthesised RNA's. At the same time, the 28S ribosomal RNA's from these two $[^{32}P]$ RNA preparations were similarly isolated and analysed for base composition.

The results (Table 8) reveal no significant difference in base composition between rapidly-labelled

RNA from infected and uninfected cells, nor do they support the hypothesis that this RNA is derived from a large part of either the host or the viral genome; the most likely explanation of the results is that this RNA, reflecting, as it does, the base composition of ribosomal RNA (Table 8), may represent a precurser of this material which may not be altered in origin after infection.

11. DNA-RNA HYBRIDISATION STUDIES.

The failure of the ^{32}P-incorporation experiment above to reveal the existence of Herpes Simplex virus DNA-like RNA as a component of 30-45S rapidly-labelled RNA in 3 hour-infected cells was possibly due to the practical difficulty of obtaining labelled RNA free from ribosomal RNA, which, under the circumstances of the experiment, would also be labelled.

An alternative method of procuring the information required is to use the DNA - RNA hybridisation tests pioneered by Hall & Spiegelman (Hall & Spiegelman [172]).

(a) Origin of 30 - 45S RNA from uninfected cells.

The first such experiment was carried out using RNA from BHK21 (C13) cells and DNA from these and from Landsdhutz ascites-tumour cells. The RNA was synthesised during a 30 minute $\left[^{14}C\right]$ uridine pulse experiment

F I G U R E 46.

Fractionation of '30 - 45S' RNA from Herpes Simplex virus-infected and uninfected BHK21 (C13) cells.

The isolated RNA preparations were analysed on sucrose gradients. '30-45S' RNA was subsequently isolated from fractions 1 to 13 (a) and fractions 1 to 12 (b).

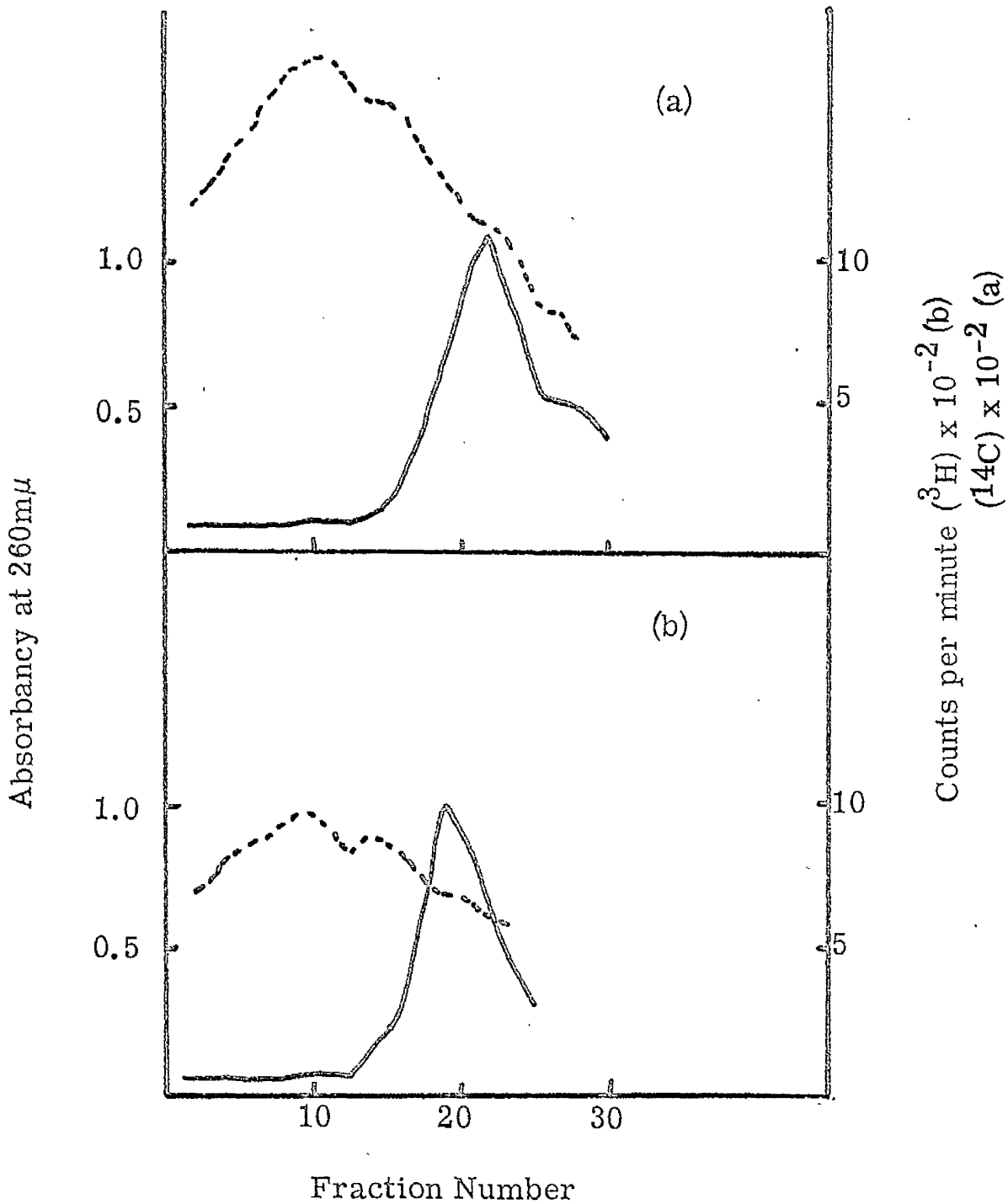
(a) uninfected, pulsed (uridine) from 3 to 3.5 hr. after infection

(b) infected, pulsed (uridine) from 3 to 3.5 hr. after infection

——— Absorbancy at 260 mμ

------ Radioactivity

FIGURE 46


(a)
(b)
1.0
0.5
10
5
10
20
30
Absorbancy at 260mμ
Counts per minute (^{3}H) x 10^{-2} (b)
(^{14}C) x 10^{-2} (a)
Fraction Number

F I G U R E 47.

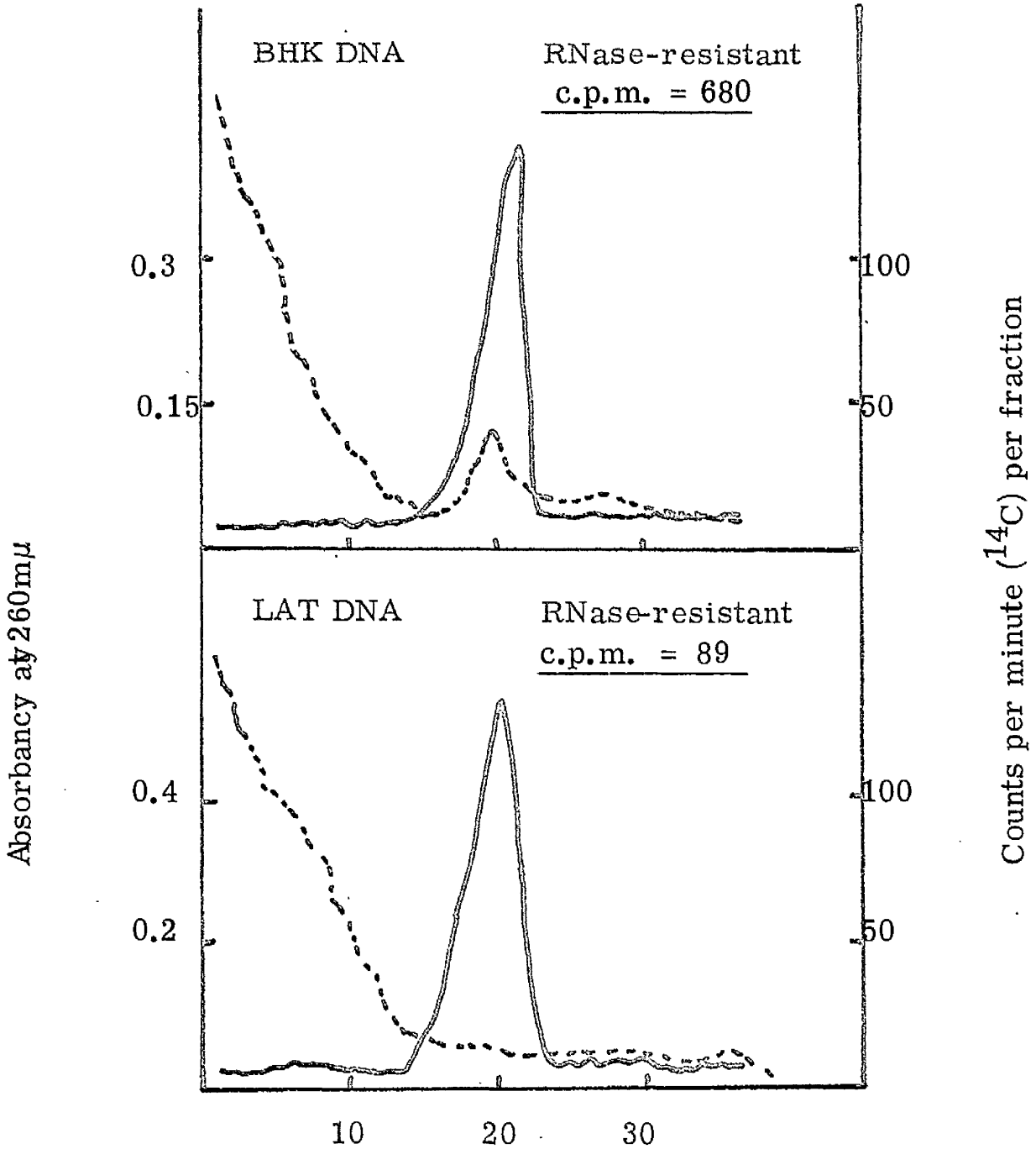
Fractionation of DNA:RNA hybridisation mixtures by equilibrium centrifugation in CsCl.

BHK21 (Cl3) DNA and Landschutz ascites tumour cell DNA were hybridised with '30-45S' RNA from BHK21 (Cl3) cells (Fig.46a). The RNase resistance of the fractions containing DNA was tested.

——— Absorbancy at 260 mμ

– – – – – Radioactivity before RNase

FIGURE 47


BHK DNA
RNase-resistant
c.p.m. = 680
0.3
0.15
100
50
LAT DNA
RNase-resistant
c.p.m. = 89
0.4
0.2
100
50
10
20
30
Fraction Number
Absorbancy at 260mμ
Counts per minute (^{14}C) per fraction

(cf. Fig. 43a); it sedimented through a sucrose gradient (Methods Sect. 7(b)) at 30 - 45S. The analysis and the fractions taken are shown in Fig. 46a. The RNA was concentrated by dialysis against 0.001M tris-HCl buffer pH 7.5 followed by lyophilisation. Total ^{14}C c.p.m. recovered were 10.5×10^3; portions containing 5000 c.p.m. were incubated in 2 x SSC with (a) 60 ug. of BHK21 (C13) DNA which had previously been denatured by heating at 100° for 10 minutes in 1/10 SSC, and cooled rapidly and (b) 60 ug. of Landshutz ascitos-tumour cell DNA treated similarly.

The incubation schedule was 70° for 5 minutes, 57° for 12 hours, then cooling to 28° over a period of 6.5 hours. Solid CsCl was added to both tubes to a final density of 1.704 gm./ml. Centrifugation followed at 33,000 r.p.m. for 3 days at 25° in the SW 39 rotor of the Spinco Model L ultracentrifuge. The gradients were harvested and analysed (Methods Sect. 8(b)) (Fig. 47). One half of each fraction was assayed for radioactivity by precipitation onto "Millipore" filter membranes followed by scintillation counting in Toluene-based scintillation fluid (Methods Sect. 6(h)(ii)). Of the remaining half of each fraction, the tubes containing DNA were pooled, diluted to 0.2M Cs^+ and treated with RNase A

F I G U R E 48.

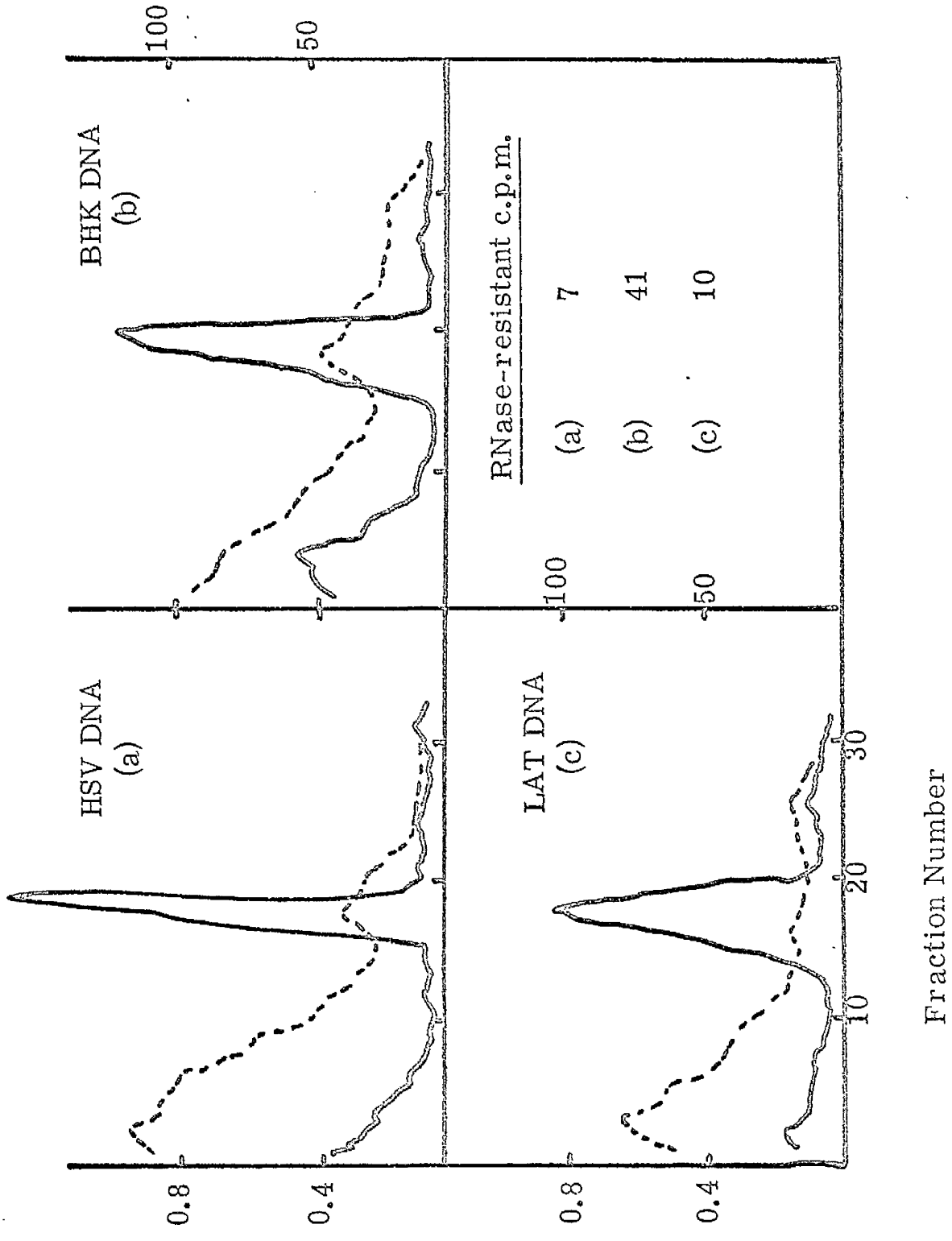
Fractionation of DNA:RNA hybridisation mixtures by equilibrium centrifugation in CsCl.

BHK21 (C13), Landsmutz ascites tumour cell and Herpes Simplex virus DNAs were hybridised with '30-45S' RNA from herpes-infected BHK21 (C13) cells (Fig.46b). The RNase resistance of the DNA-containing fractions was tested.

——— Absorbancy at 260mμ

– – – – Radioactivity before RNase

FIGURE 48


Counts per minute (^{3}H)
100
50
BHK DNA
(b)
RNase-resistant c.p.m.
(a) 7
(b) 41
(c) 10
100
50
HSV DNA
(a)
LAT DNA
(c)
0.8
0.4
0.8
0.4
10
20
30
Absorbancy at 260mμ
Fraction Number

(15 ug./ml. at 25° for 15 minutes). Acid-insoluble material surviving this treatment was collected on "Millipore" membranes and counted as described above. The results are presented in Fig. 47: the radioactive RNA associated with DNA from BHK21 (C13) cells but not with that from Landsshutz ascites-tumour cells. That the association is a strong one is emphasised by its resistance to the RNase action. This evidence suggests that 30 - 45S rapidly-labelled RNA from BHK21 (C13) cells is transcribed specifically from BHK21 (C13) cell DNA.

11.

(b) Origin of 30 - 45S RNA from infected cells.

30 - 45S rapidly-labelled RNA (from Results Sect. 9a) was obtained from BHK21 (C13) cells pulsed 3 to 3.5 hours after infection with Herpes Simplex virus (Fig. 43b). The RNA (22 x 10^3 c.p.m.) was divided into three portions and incubated for annealing purposes as above, with 60 ug. of (a) Herpes Simplex virus DNA, heat-denatured in 1/50 SSC for 10 minutes at 100° (b) BHK21 (C13) DNA and (c) Landsshutz ascites-tumour cell DNA, both denatured as before. One quarter of each incubation mixture was treated with RNase A, while the remainder was centrifuged through a CsCl gradient as above. The results (Fig. 48) suggest that the RNA formed a hybrid, albeit to a small extent, only with Herpes Simplex virus DNA or BHK21 (C13)

TABLE 9.

The design of an experiment to investigate the synthesis of RNA in BHK21 (C13) cells before and after Herpes Simplex virus infection.

All samples were pulsed with radioactive uridine. The experiment is described in Results sect.12a.

TABLE 9

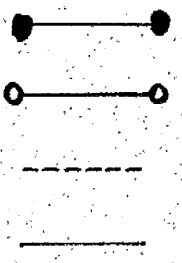
TABLE 9

Time	15'	30'	45'
Treatment	pulse I1	pulse I2	pulse C1 harvest I1 pulse I3
60'	75'	90'	105'
pulse I4 harvest I2	pulse I5 harvest I3,C1	harvest I4	harvest I5
3hr. 15'	3hr. 45'	5hr. 15'	5hr. 45'
pulseI6,C2	harvest I6,C2	pulse I7	harvest I7
7hr. 15'	7hr. 45'		
pulseI8,C3	harvest I8,C3		

F I G U R E 49.

The kinetics of RNA production in Herpes Simplex virus-infected BHK21 (Cl3) cells in relation to the production of virus particles.

The RNA was isolated from infected and uninfected cells after a 30 minute exposure to radioactive uridine at the indicated times.



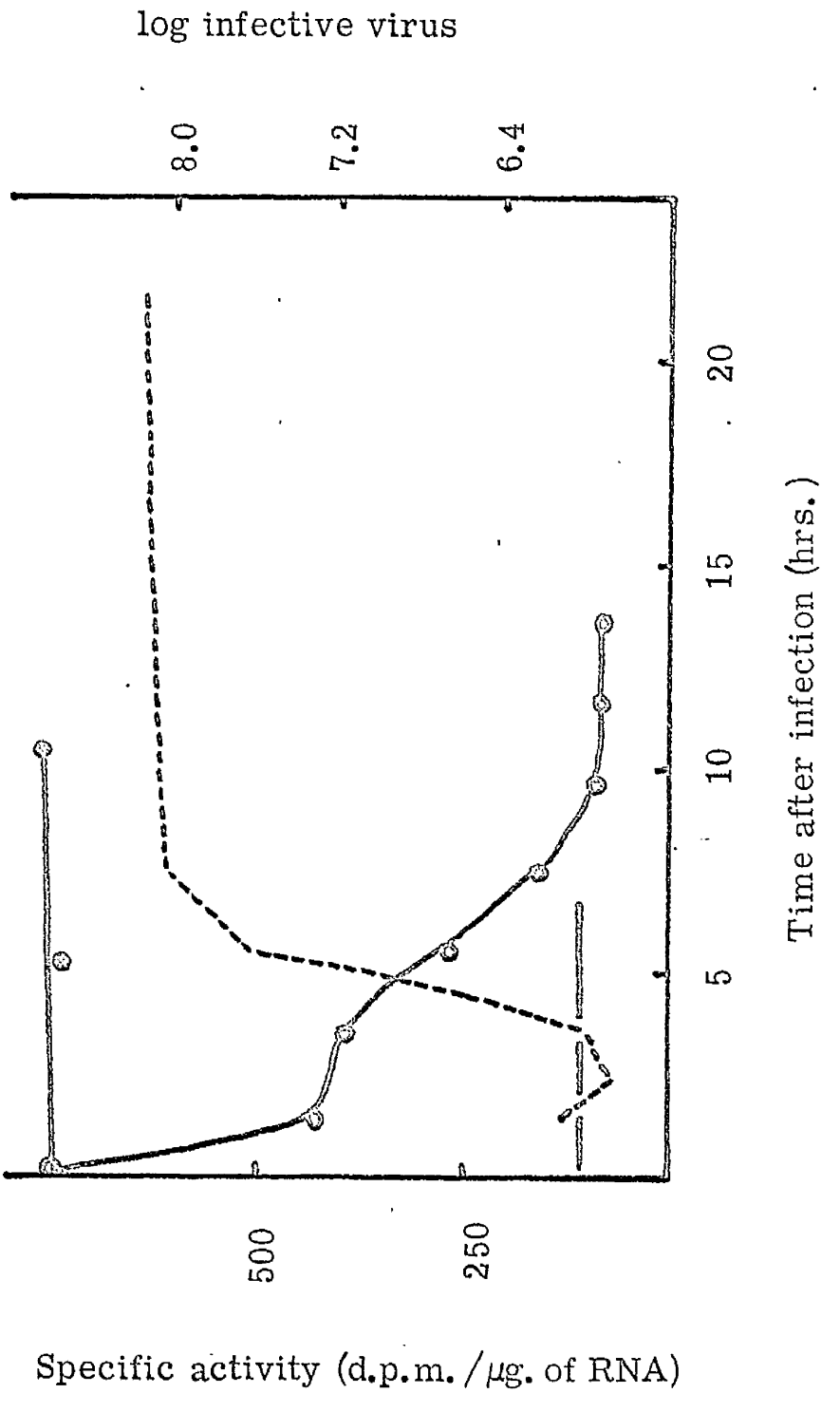
RNA synthesis (control)

RNA synthesis (Infected)

Infectivity

Infective centres

FIGURE 49


log infective virus
8.0
7.2
6.4
5
10
15
20
Time after infection (hrs.)
500
250
Specific activity (d.p.m./μg. of RNA)

DNA. The result of RNase treatment (Fig. 48) was loss of the Herpes Simplex virus DNA - RNA hybrid but not of the BHK21 (C13) DNA - RNA hybrid. While it should be stressed that this amount of hybridisation is extremely small, it suggests that while host-specified synthesis of RNA at 30 - 45S continues after infection, no comparable size of virus-specified RNA is apparent at 3 hours after infection.

12. RNA SYNTHESIS AFTER INFECTION-EXTENDED TIME SCALE.

(a) Studies in RNA synthesis after infection of BHK21 (C13) cells with Herpes Simplex virus have covered the period 1 - 3 hours after infection (Results Sect. 9, 10). Virus coded RNA may be produced immediately following infection for a short time or arise at times later than 3 hours. Table 9 describes the design of an experiment to test these two possibilities. A 30 minute pulse time was retained, but $[^{14}C]$ uridine was used in control uninfected cultures and $[^{3}H]$ uridine in infected ones. RNA was isolated and fractionated on sucrose gradients (Methods Sect. 7 (b)).

Fig. 49 relates the synthesis of RNA in control and Herpes Simplex virus infected BHK21 cells as a function of time. This figure also gives information on the production of new virus particles during the experiment

F I G U R E 50.

RNA synthesis in BHK21 (C13) cells after Herpes Simplex virus infection.

The RNA, isolated after a 30 minute pulse of radioactive precursor, was fractionated on a sucrose gradient. The elution patterns are shown.

(a) Infected, pulsed 0.75 - 1.25hr.

(b) Infected, pulsed 1.25 - 1.75 hr.

(c) Infected, pulsed 3.25 - 3.75 hr.

(d) Infected, pulsed 5.25 - 5.75 hr.

(e) ^{3}H - infected, pulsed 7.25 - 7.75hr.
^{14}C - control, pulsed 7.25 - 7.75 hr.

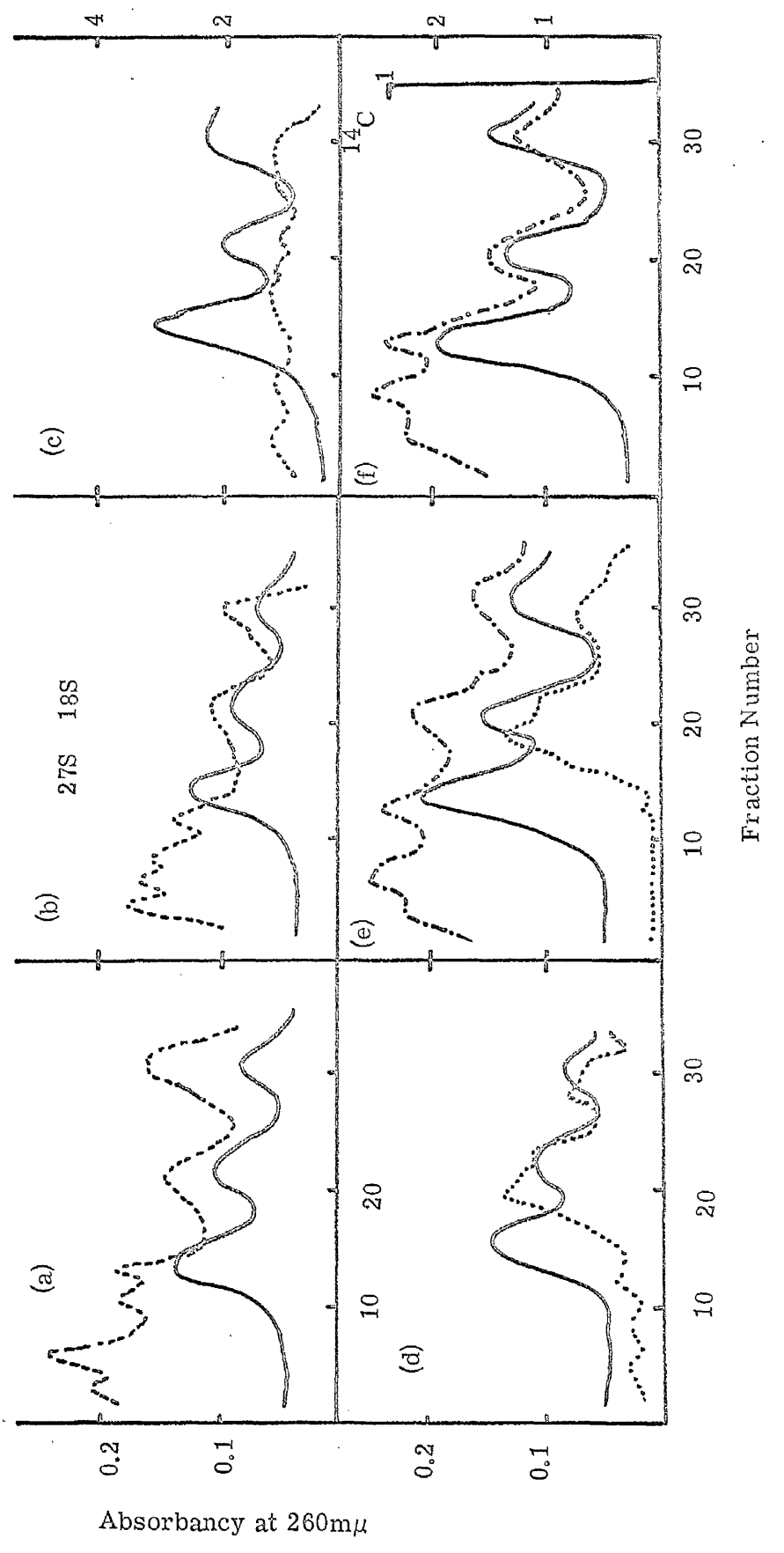
(f) Control, pulsed 0 - 0.5 hr.

——— Absorbancy at 260 mu

^{3}H ..... Radioactivity

^{14}C -.-.-.-

FIGURE 50


Counts per minute (^{3}H) x 10^{-2}
(a)
(b)
27S 18S
(c)
(d)
(e)
(f)
^{14}C
Absorbancy at 260mμ
Fraction Number

as revealed by Infective Centre and Infectivity assays (Russell et.al. [148]). According to this last criterion, mature Herpes Simplex virus was produced first at 3 - 4 hours and reached peak production at 8 hours after infection. During this time interval, RNA synthesis in infected cells fell exponentially to 60% of the control level by 1.5 hours, and retained this level until approximately 3.5 hours. A further steady fall took place up to 9 hours when 12% of the control level was reached, and this level was maintained for at least a further 5 hours.

Sucrose gradient analyses of the RNA's were then carried out (i) to identify the cellular rapidly-labelled RNA fractions whose synthesis declines after herpes infection and (ii) to characterise any new RNA arising from virus-directed transcription. The results are given in Fig. 50. Analysis of RNA isolated immediately after infection is not included because it differs from control analysis only quantitatively (see Fig. 49). The absorbancy patterns in the density gradient analyses indicate the position of the characteristic RNA species derived from ribosomes (27S and 18S) and of 4S RNA. Incorporation of [^{14}C] uridine into the rapidly-labelled RNA of uninfected cells gave rise to a pattern which

characteristically showed two main peaks of high specific activity at 45S and 35S, a peak in the 4 to 6S region and further peaks of incorporation coincident with the ribosomal RNA components (Fig. 50e,f). Fig. 50a represents the analysis of RNA from cells infected with herpes virus and then exposed to [^{3}H] uridine from 0.75 to 1.25 hours. Qualitatively, the level of incorporation into RNA had not altered appreciably from that found in uninfected cells (Fig. 50e,f), but quantitatively it had fallen, in agreement with earlier work. (Experiment 9a).

Fig. 50b, which refers to the sample pulsed from 1.25 to 1.75 hours, gave the first indication of a qualitative change in the 20S region of the sucrose gradient. Infected cells pulsed with [^{3}H] uridine from 3.25 to 3.75 hours showed a marked decrease in incorporation into 45S and 35S RNA (Fig. 50c), while a broad peak of rapidly-labelled RNA at 20S had become apparent: no comparable peak at 20S was present in uninfected cells. Two hours later (5.25 to 5.75 hours) RNA synthesis had become confined to this broad peak at 20S, and, to some extent, to 4S material, while 45S and 35S RNA synthesis had fallen to a very low level (Fig. 50d). In the 7.25 hours to 7.75 hours sample,

synthesis of 45S and 35S RNA was not detected, and the rates of synthesis of 20S RNA and 4S RNA had fallen relative to the earlier time sample (Fig. 50c, ^{3}H incorporation).

An experiment similar to this has shown that the pattern of synthesis of rapidly-labelled RNA in cells infected for 13.5 hours is identical to that observed at 7.25 to 7.75 hours (Fig. 50e, ^{3}H incorporation) but with a lower rate of synthesis (cf. Fig. 49).

The initial decline in incorporation shown in Fig. 49 is probably owing to the fall in synthesis of 45S and 35S RNA apparent from Fig. 50, while the later fall in the rate of RNA synthesis may represent a decrease in the extent of synthesis of the 20S RNA, or, more likely, it may reflect the metabolic death of a proportion of the infected cells.

12.

(b) Origin of the 20S RNA formed after infection.

It has been established by DNA-RNA Hybridisation techniques that the 30-45S rapidly-labelled RNA in uninfected and 3 hours-infected BHK21 (C13) cells is cell-specified (Results Sect. 11a,b). It was important therefore, using the same technique, to

investigate the specific genetic origin of the 20S RNA (Fig. 50e) formed after infection of these cells with Herpes Simplex virus.

50 µg. portions of heat-denatured BHK21 (C13) Landshutz ascites-tumour and Herpes Simplex virus DNA's were individually incubated under standard conditions with 20S RNA (10^4 c.p.m.) from 7.25 to 7.75 hours after infection. Incubation mixtures were held at 75^0 for 10 minutes, 65^0 for 2 hours and then cooled to 25^0 over a period of 12 hours. CsCl gradient centrifugation was carried out as before (Methods Sect. 8(b)) on each mixture. Fractions harvested from the bottom of each gradient (containing unhybridised RNA) were pooled, and likewise the fractions from the middle of the gradient (containing the DNA peak) were pooled. Each pool was diluted to 0.2M CsCl. One quarter of each pool was then taken for measurement of acid-insoluble radioactivity while the remainder was treated for 35 minutes at 37^0 with RNase A at a concentration of 7 µg. per ml. Ribonuclease-resistant material was collected on disks of Teflon-coated glass fibre paper using 5% w/v trichlor-acetic acid and measured for radioactivity in the liquid scintillation spectrometer (Methods Sect. 6(h)(ii)).

TABLE 10.

Hybrid formation between 20S RNA from cells infected with Herpes Simplex virus and DNA from various sources.

The hybridisation procedure is described in Methods sect.9a.

TABLE 10

Source of DNA	Fractions pooled after CsCl gradient Centrifugation	Total d.p.m.(^{3}H) in pooled fraction	
		Before RNase treatment	After RNase treatment
BHK 21 (C13) cells	Bottom 5 fractions	390	0
	A_{260} peak fractions	262	40
Landshutz ascites-tumour cells	Bottom 5 fractions	675	6
	A_{260} peak fractions	75	0
Herpes Simplex virus	Bottom 5 fractions	375	0
	A_{260} peak fractions	225	107

Table 10 shows that most of the 20S RNA which associated with the DNA of herpes virus withstood the action of RNase. Some of the 20S RNA also formed RNase-resistant associations with BHK21 (C13) DNA, though not with Landshutz ascites-tumour cell DNA.

Hybrid formation with BHK21 (C13) DNA was to be expected in this particular experiment as the subsequent sedimentation analysis at this sample time showed that some 45S and 35S material was still being synthesised. (This suggests that not all of the cells were infected at the beginning of this experiment). Corroborative evidence of hybridisation between 20S RNA from infected cells and DNA of Herpes Simplex virus has also been obtained using the experimental procedure of (Becker & Joklik [335]). This consists of assaying for RNase-resistant but unfractionated hybrid material.

It is concluded that BHK21 (C13) cells, 7.25 to 7.75 hours after infection with herpes virus, synthesise virus-specified RNA which sediments as a broad band at 20S: it is not possible to rule out completely the possibility that some host-directed 20S RNA synthesis takes place under these conditions.

TABLE 11.

Synthesis of ribosomal RNA in uninfected and Herpes Simplex virus infected BHK21 (C13) cells.

Cells were exposed to [^{14}C] uridine for 2 hr. before infection and [^{3}H] uridine for the shown times after infection. Control cells were not infected.

Specific activity is c.p.m. per unit of absorbancy at 260 mμ

TABLE II

Time after Infection (hr.)	Specific Activity of Ribosomal RNA				Infected / Control	
	Control		Infected			
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
0.5 - 2.0	2.2	1.2	1.6	1.3	0.73	1.08
2.0 - 3.5	1.7	1.3	0.92	1.35	0.54	1.03
3.5 - 6.0	1.8	1.4	0.7	1.45	0.39	1.03

13. RIBOSOMAL RNA

(a) Effect of Herpes Simplex virus infection on ribosomal RNA synthesis.

In the course of the last few experiments investigating the synthesis of rapidly-labelled RNA in uninfected and herpes-infected BHK21 (C13) cells, it was observed that a little ribosomal RNA was elaborated during a 30 minute pulse in uninfected cells. The stability of this RNA after infection and the effect of infection on its synthesis is of interest and, accordingly, was investigated. However, longer exposure times to radioactive precursor were used, ribosomal RNA having a longer turnover time than the rapidly-labelled fraction studied previously.

BHK21 (C13) cells were exposed to $[^{14}C]$ uridine for 2 hours before infection with Herpes Simplex virus. Infected and control cell samples were exposed to $[^{3}H]$ uridine from 0.5 to 2.0 hours, 2.0 to 3.5 hours, and 3.5 to 6.0 hours. Each sample was harvested and fractionated for RNA as before. (Results Sect. 9a). Ribosomal RNA was separated by sucrose gradient analysis of the total cell RNA (Methods Sect. 7(d)).

The results (Table 11) suggest that ribosomal RNA synthesised in these cells before infection was

stable up to 6 hours after infection ^{14}C specific activities); and that ribosomal RNA continued to be synthesised in the cells following infection. The rate of synthesis falls rapidly as infection proceeds and reaches 35% of the control level in the period 3.5 to 6.0 hours after infection ^{3}H specific activities).

The rate of ribosomal RNA synthesis in the period immediately following plating of the control (uninfected) cells is higher than that found subsequently. This may be a consequence of increased metabolic activity in the cells following dispersal and subsequent plating on glass. The total amount of RNA did not vary significantly among samples.

13.

(b) Sedimentation Coefficient measurements on ribosomal RNA.

The RNA isolated in the course of the last experiment was used to determine the absolute S value of the ribosomal RNA species in BHK21 (C13) cells (a) under several conditions of ionic strength and (b) before and after infection.

Ribosomal RNA (40µg./ml.) from uninfected cells was dissolved in (a) 0.14M NaCl (b) 0.14M NaCl/5 x 10^{-3}M $MgCl_2$ or (c) 0.14M NaCl/5 x 10^{-3}M EDTA and centrifuged

F I G U R E 51.

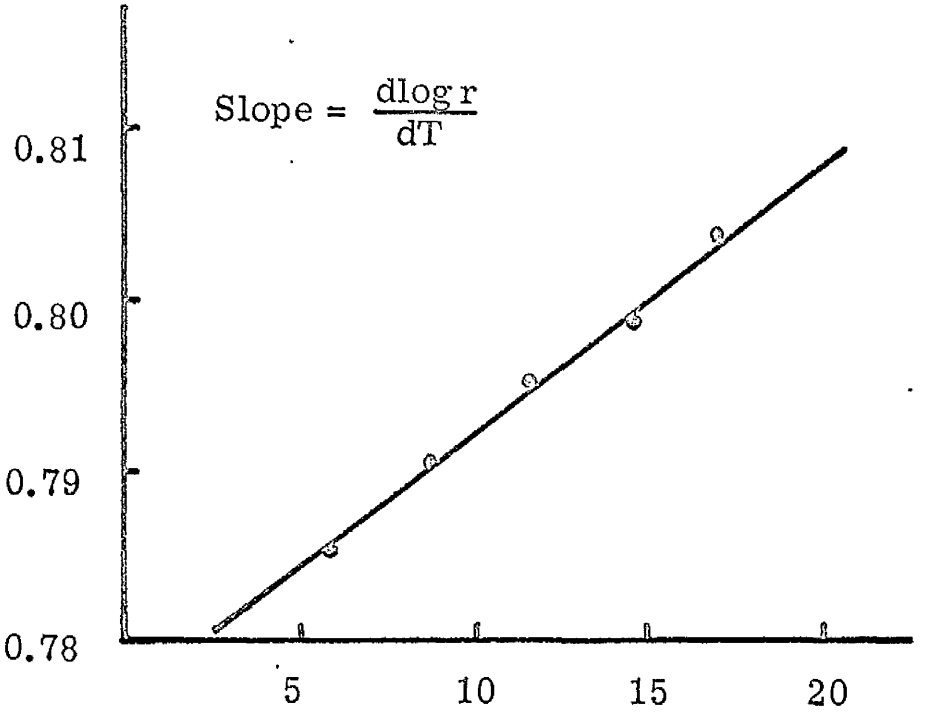
Calculation of the Sedimentation Coefficient of a ribosomal RNA species from BHK21 (C13) cells.

x = distance of the moving boundary of solute from the centre of rotation of the rotor.

w = rotor speed in radians per second

The Spinco model E ultracentrifuge was used.

FIGURE 51



$$S = \frac{1}{w^2} \times \frac{d\log r}{dT}$$

$$= \frac{0.0305}{20} \times 17.47 \times 10^{-10}$$

$$= \underline{26.7 \times 10^{-13}} \ \text{sec}^{-1}$$

TABLE 12.

The effect of Mg^{++}, EDTA and Herpes Simplex virus infection on the sedimentation coefficients of ribosomal RNA from BHK21 (C13) cells.

TABLE 12

RNA isolated from	Solvent	Sedimentation Coefficients of Ribosomal RNA	
BHK 21 (C13) cells	0.14M NaCl $5x10^{-3}$M $MgCl_2$	32S	18.6S
BHK 21 (C13) cells	0.14M NaCl $5x10^{-3}$M EDTA	27S	17.5S
BHK21 (C13) cells	0.14M NaCl	27.5S	18S
6hr. Herpes-infected BHK 21 (C13) cells	0.14M NaCl	27.7S	18S

at 44,770 r.p.m. in the Spinco Model E ultracentrifuge. Ultraviolet absorption photographs of the boundaries of solute were taken at 8 minute intervals after the start of the run and the photographs were traced out on graph paper using the Beckman Analytrol Microdensitometer. RNA from control cells and 6.0 hour-infected cells were dissolved in 0.14M NaCl and treated exactly as detailed above.

Fig. 51 details a representative calculation of sedimentation coefficient in this system: all samples were analysed similarly. Table 12 gives the results of the analyses. It shows that the ribosomal RNA's from BHK21 (C13) cells, under standard conditions, have S values of 27S and 18S and that these values are not affected by Herpes Simplex infection. It is also clear that the addition of Mg^{2+} at 5×10^{-3}M to the system increases the S value of both ribosomal RNAs significantly: this effect is probably due to aggregation. EDTA at a similar concentration has no effect.

14. <u>CALCULATION OF THE S VALUES OF RAPIDLY-LABELLED RNA.</u>

(a) Insufficient amounts of rapidly-labelled RNA are available on which to carry out the above procedure. Calculation of S values, albeit approximate, of this

F I G U R E 52.

Sedimentation Coefficients of RNA species from Herpes Simplex virus-infected and uninfected BHK21 (C13) cells.

The values shown were obtained using the method of Martin & Ames [364]

——— Absorbancy at 260mμ

------ Radioactivity (uninfected)

—.—.— Radioactivity (infected)

F I G U R E 53.

Fractionation of a DNA:sRNA hybridisation mixture in a CsCl density gradient at equilibrium.

Herpes Simplex virus and BHK21 (C13) cell DNAs were hybridised with $[^{32}P]$sRNA from herpes-infected cells. After CsCl fractionation, one half of each fraction was treated with RNase. Both fractions were subsequently assayed for radioactivity

——— Absorbancy at 260mμ

—.—.— Radioactivity before RNase

------ Radioactivity after RNase

FIGURE 52

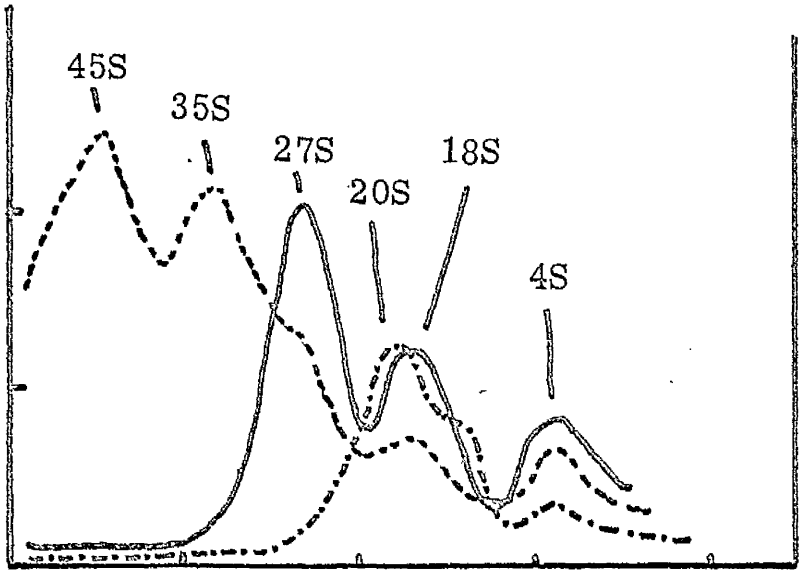
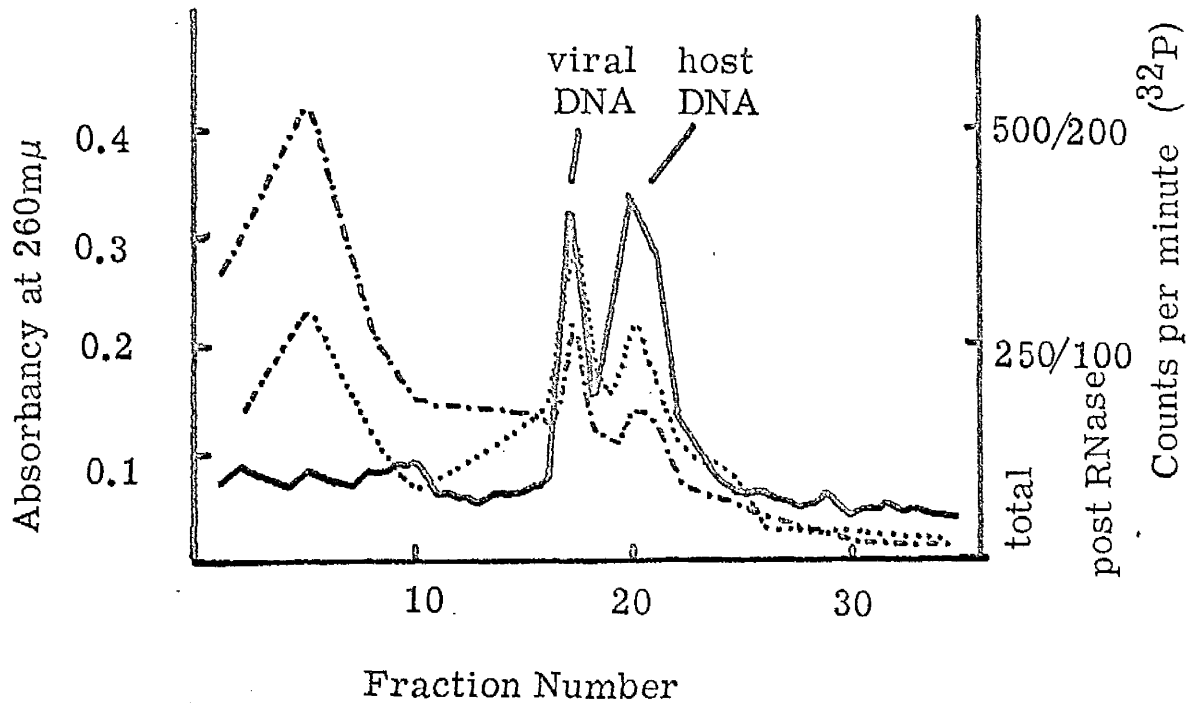


FIGURE 53



F I G U R E 71.

Synthesis of RNA during a one or two hour pulse period.

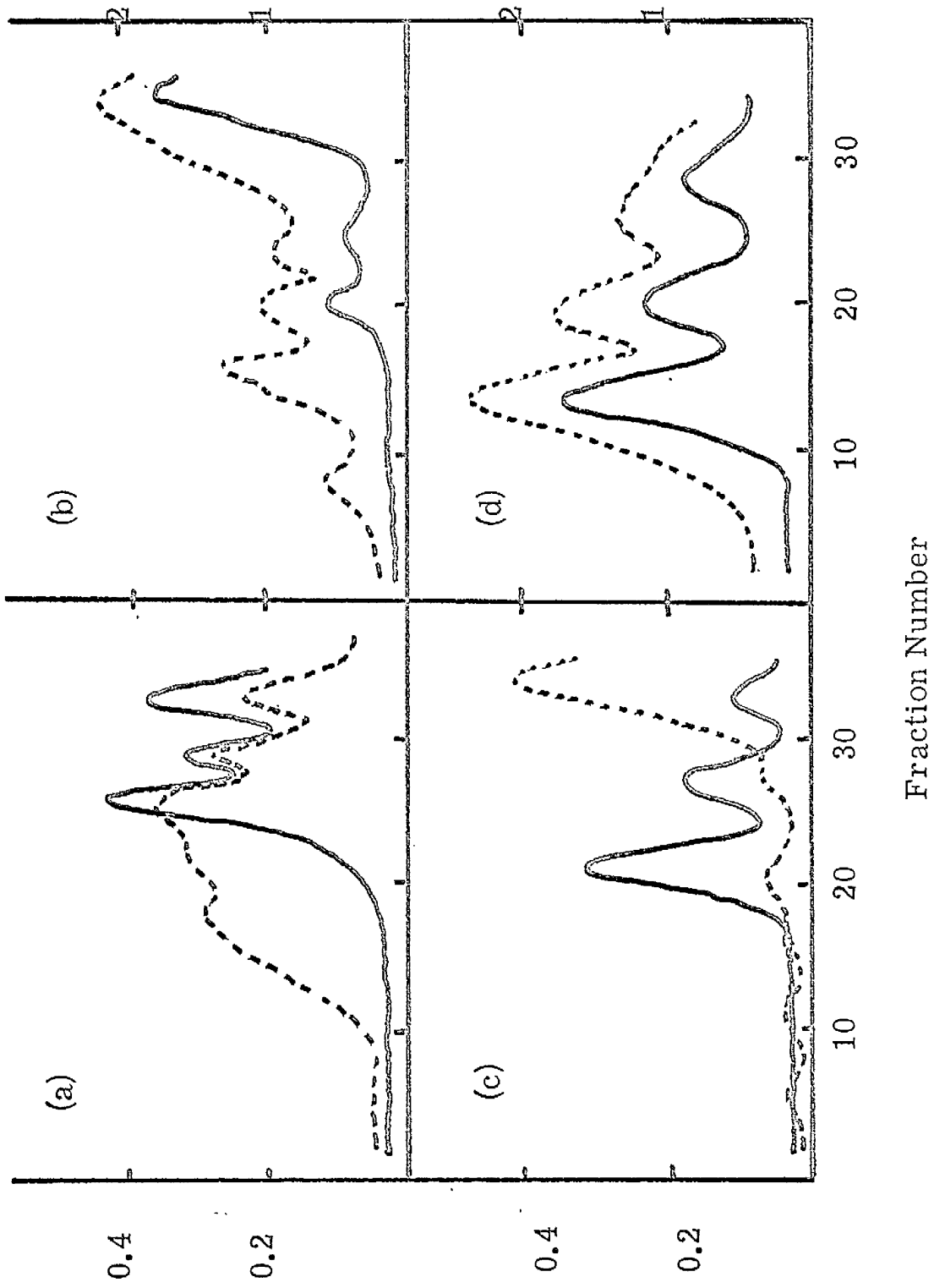
The following RNA samples were fractionated on sucrose gradients:

(a) from BHK21 (C13) cells pulsed with uridine for 1 hr. (b) from the nuclear fraction of cells infected with Herpes Simplex virus and pulsed between 6 & 7 hr. after infection. (c) from the cytoplasmic fraction of cells as (b). (d) from cells pulsed for 2 hr.

_____ Absorbancy at 260 mμ

_ _ _ _ _ Radioactivity

FIGURE 71


Counts per min. per fraction (b, c x 10^{-2}; a, d x 10^{-3})
(a)
(b)
(c)
(d)
0.4
0.2
0.4
0.2
10
20
30
10
20
30
Fraction Number
Absorbancy at 260mμ

material may be accomplished, however, using the technique of (Martin & Ames [364]). This method involves comparison of the extent of movement through a sucrose gradient of unknown material with that of material of calculated S value: this depends on the materials possessing the same partial specific volume. The calculation is one of simple proportion, and the reference material used is ribosomal RNA. The results are shown in Fig. 52.

14.

(b) Synthesis of labelled RNA in cells exposed to [^{3}H] uridine for one and two hour periods.

Two batches of BHK21 (C13) cells were exposed to [^{3}H] uridine (4 uC/ml.) for one and two hours respectively before harvesting and extraction of RNA (Methods Sect. 5(b)). The results of a sucrose gradient analysis (Methods Sect. 7(b)) of these RNAs is given in Fig. 71a (one hour pulse) and d (two hour pulse). The analysis of the one hour pulsed material was purposely constructed to give information on the possibility of there being very high molecular weight rapidly-labelled RNA present (see Results Sect. 8g). This fractionation consisted of a two hour centrifugation at 33,000 r.p.m. in the S W 40 rotor of the Griffin

Christ ultracentrifuge.

A similar analysis was applied to RNA of cytoplasmic and nuclear fractions (Methods Sect. 6(f)) from BHK21 (C13) cells, infected with herpes virus, and pulsed with [^{3}H] uridine between 6 and 7 hours after infection.

The results are shown in Fig. 71b (nuclear) and c (cytoplasmic) respectively, and indicate that while most of the RNA synthesised in this time is retained in the nucleus (both host and virus RNA) a little, largely 4S RNA, has either been formed in or migrated to the cytoplasm.

TRANSFER RNA.

In the course of earlier work investigating the synthesis of RNA in BHK21 (C13) cells infected with Herpes Simplex virus, it was consistently observed (e.g. Fig. 50) that production of RNA sedimenting in the 4S region of a sucrose gradient continued after infection. This synthesis appears to diminish as infection proceeds, and, like post-infective ribosomal RNA synthesis, may represent residual metabolic activity of dying cells. Alternatively, it could reflect transcription of the virus genome, as was observed in the case of production of 20S RNA (Fig. 50).

15.

Some purely theoretical arguments lend support to this latter hypothesis. The large difference in G plus C content between virus (68%) and host DNA (41%) suggests the following consideration. Unless there is extensive redundancy, the population of tRNA molecules normally present in the host cell must be optimally adjusted to the requirements of messenger RNA transcribed from cell DNA of 41% G plus C; it cannot at the same time be optimally adjusted to meet the translation requirements of herpes virus messenger coded for by

DNA of 68% G plus C. This consideration applies particularly at the time of maximum synthesis of virus-specified proteins (Russell et.al. [148]).

Josse et.al. (Josse et.al. [38]) established by nearest neighbour analysis of DNA that the four bases occur next to one another in the sixteen possible doublet arrangements not at random, but in a pattern of frequencies unique for every DNA. In animal cells, the doublet CpG occurs with particularly low frequency. Doublet frequencies based on published (Swartz et.al. [36]) and unpublished (Subak-Sharpe et.al. [318]) nearest neighbour analyses of mammalian and Herpes Simplex virus DNA respectively have been compared. It was found that the frequencies of the four doublets GpC, CpG, GpG and CpC found in mammalian cell DNA represent 36%, 9%, 44% and 41% of the respective frequencies observed for Herpes Simplex DNA. Since all or nearly all codons must be assigned, (Woese [323]) this suggests that of the 64 tRNA molecules corresponding, in the host cell, to all the possible triplet codons, the 24 tRNA molecules which correspond to the codons incorporating the CpC, GpG, CpG and GpC doublets will be in short supply, especially at the time of extensive viral protein synthesis.

The eight triplets containing the CpG doublet, in particular, appear to be poorly represented. However, if we suppose that the invading virus genome itself contains genetic information for certain tRNAs, then the tRNA population following infection would change in a direction favouring the requirements for virus protein synthesis.

On the basis of these considerations the following experiments were undertaken.

(a) <u>The genetic origin of '4S RNA' in Herpes Simplex infected cells.</u>

The first exploratory experiment carried out was a test for homology between labelled sRNA from control and herpes-infected BHK21 (C13) cells, and the host and virus DNA. Four 80 oz. bottles of BHK21 (C13) cells were infected with Herpes Simplex virus at a multiplicity of exposure of 10 P.F.U./cell. These, and four uninfected bottles were immediately exposed to 1mC of ^{32}P-orthophosphate each. After 8.5 hours, the medium was removed and sRNA was extracted from infected and uninfected cells by the method of Hoagland et.al. (Hoagland <u>et.al.</u>[344]), except that bentonite was employed to remove ribonuclease.

Following the procedure of Giacomoni & Spiegelman (Giacomoni & Spiegelman [353]), 50 µg. of heat-denatured

BHK21 (C13) DNA or Herpes Simplex DNA were incubated with 10 ug. of sRNA from infected cells. The mixture was then fractionated on a CsCl gradient and harvested by the standard procedure (Methods Sect. 8(b), 9(b)). After absorbancy measurement at 260mμ, one half of each fraction was treated with RNase at 15 μg./ml. for 15 min. at 30° in the presence of 0.2M Cs^+. Both parts of each fraction were then precipitated with acid and assayed for radioactivity using teflon-coated glass-fibre paper in the standard way (Methods Sect. 6(h)(ii)).

Fig. 53 shows the result of this analysis. It indicates that sRNA from Herpes Simplex infected cells forms an RNase-resistant hybrid with the viral DNA, and also, to a lesser extent, with BHK21 (C13) DNA. In an identical analysis using Landshutz ascites-tumour cell DNA in place of BHK21 (C13) DNA, no hybridisation was observed.

(b) Purification of infected cell sRNA.

The sRNA shown in the last experiment to be capable of specific binding to DNA had been purified in such a way that, in addition to tRNA, it may have contained fragments of other RNA species, themselves capable of hybridisation.

F I G U R E 5/4.

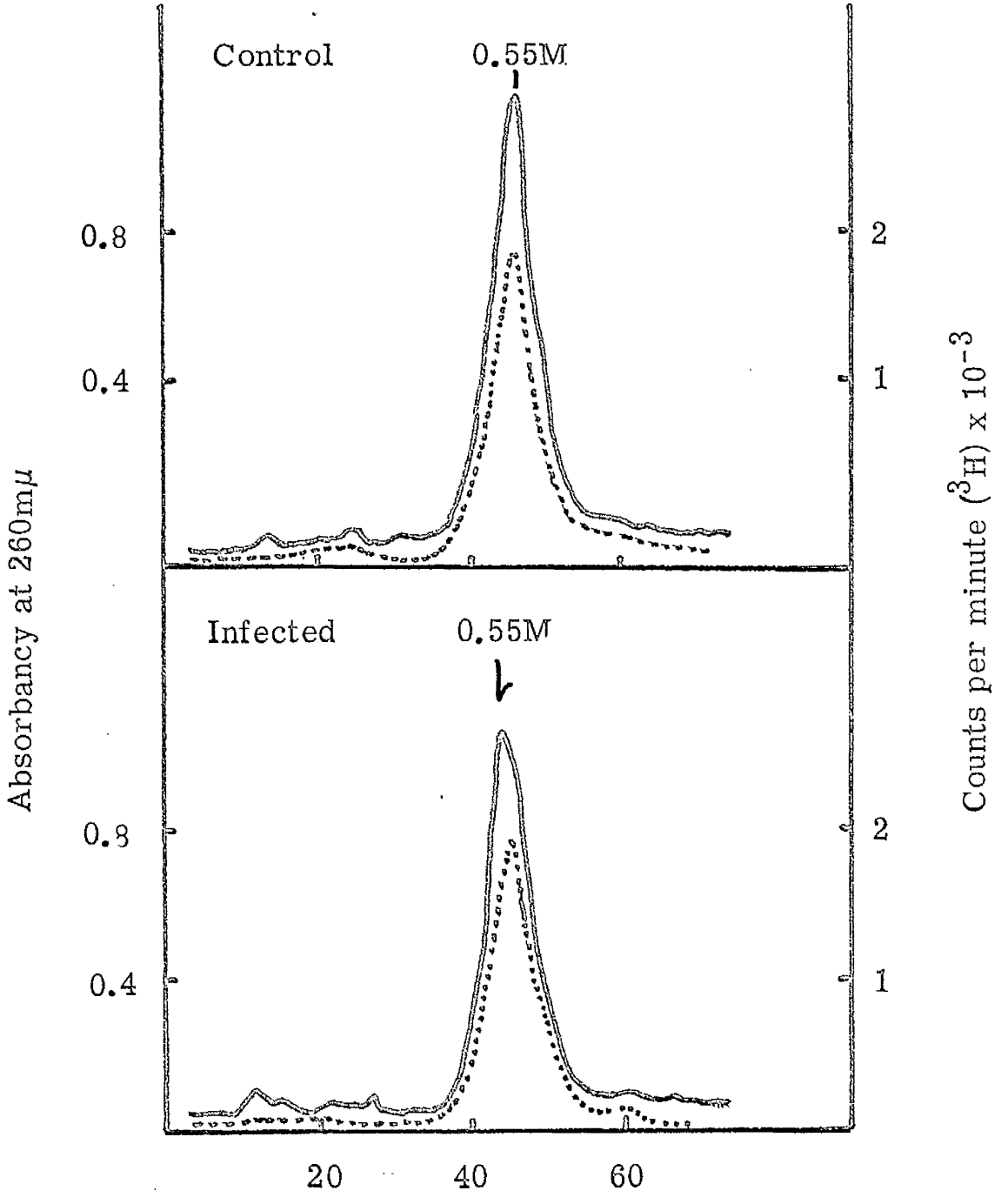
Chromatography of sRNA on columns of DEAE - cellulose.

sRNA prepared from Herpes Simplex virus-infected and uninfected BHK21 (C13) cells was applied to and eluted from a column of DEAE-cellulose. Elution was obtained using a linear gradient of NaCl. 3ml. fractions were collected.

——— Absorbancy at 260mμ

- - - Radioactivity

FIGURE 54


Control
0.55M
Infected
0.55M
0.8
0.4
2
1
0.8
0.4
2
1
20
40
60
Absorbancy at 260mμ
Counts per minute (^{3}H) x 10^{-3}
Fraction Number

F I G U R E 55.

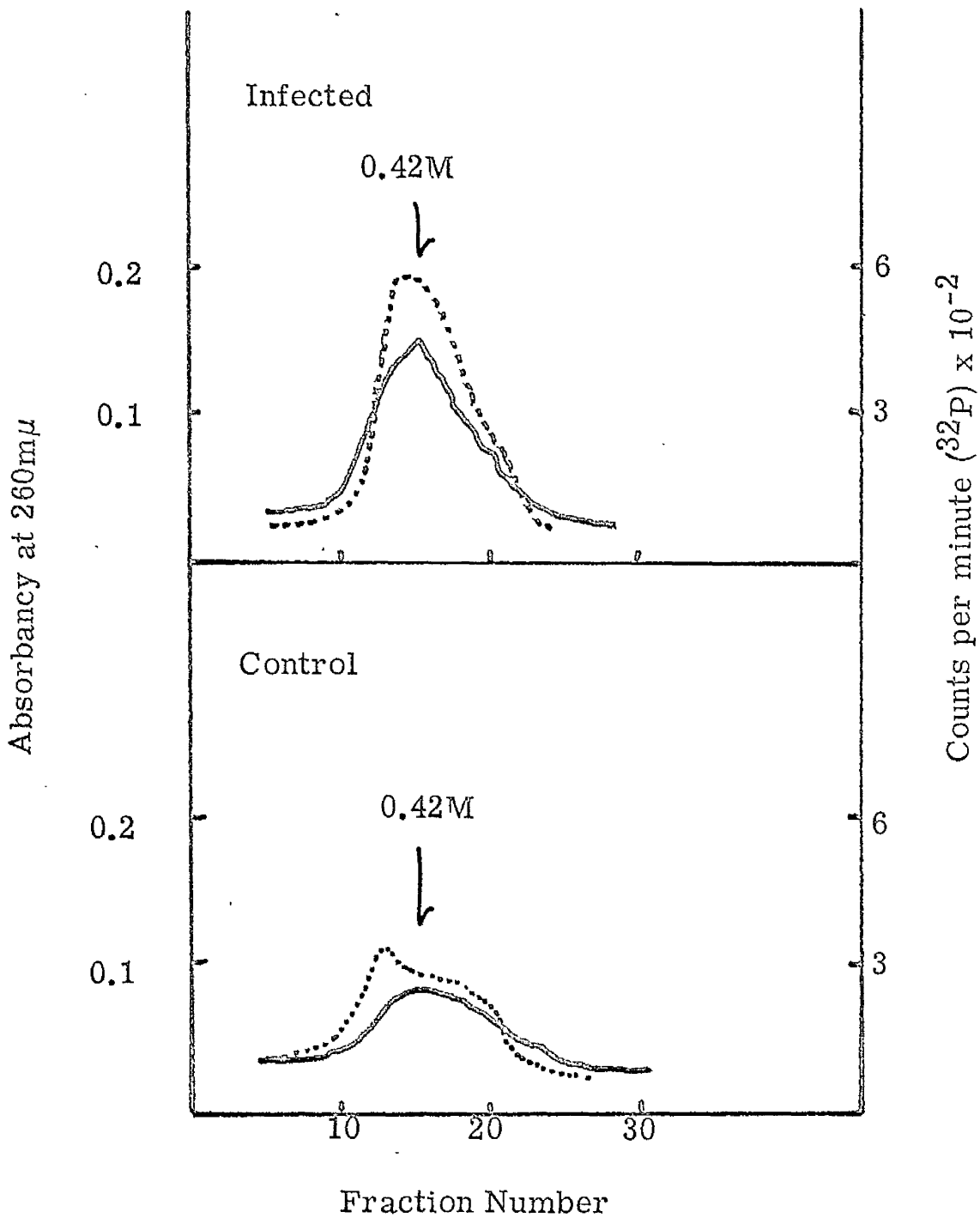
Fractionation of sRNA on columns of MAK.

sRNA prepared from Herpes Simplex virus-infected and uninfected MK21 (Cl3) cells was applied to and eluted from an MAK column. The elution was achieved with a linear NaCl gradient and 3 ml. fractions were collected.

——— Absorbancy at 260 mμ

– – – – Radioactivity

FIGURE 55


Infected
0.42M
0.2
0.1
6
3
Control
0.42M
0.2
0.1
6
3
10
20
30
Absorbancy at 260mμ
Counts per minute (^{32}P) x 10^{-2}
Fraction Number

No method exists, however, for the unequivocal purification of tRNA; nevertheless moderately rigorous methods of purification were employed in the hope of revealing any contaminants.

Accordingly, control and Herpes Simplex infected cell sRNAs were further purified on columns of DEAE - cellulose (Methods Sect. 2(d)) from which both preparations were eluted at 0.55M with a linear NaCl gradient (Bell et.al.[361]). Little or no contamination was detectable in either sRNA preparation (Fig. 54).

This purified sRNA was fractionated by analytical-scale electrophoresis on agarose gel under conditions in which contaminating cell RNA of size larger than 4S would be apparent (Methods Sect. 7(c)). No such contamination could be detected in material from control or infected cells.

More critical fractionation of sRNA than that obtainable using DEAE - cellulose may be achieved on MAK columns. Qualitatively, the elution patterns of infected and uninfected cell sRNAs (Fig. 55) were similar to those described by other workers for tRNA (Yamane et.al.[240]) and, once more, little or no contamination of the main mass of RNA with fragments was visible.

F I G U R E 56.

CsCl equilibrium centrifugation of DNA:sRNA hybridisation mixtures from control and infected cells.

Herpes Simplex virus and BHK21 (C13) cell DNAs were hybridised with purified sRNA from infected and uninfected cells. The mixtures were analysed in CsCl density gradients and each fraction was treated with RNase prior to radioactivity measurements.

(a) Cell DNA with cell sRNA:

(b) Virus DNA with cell sRNA:

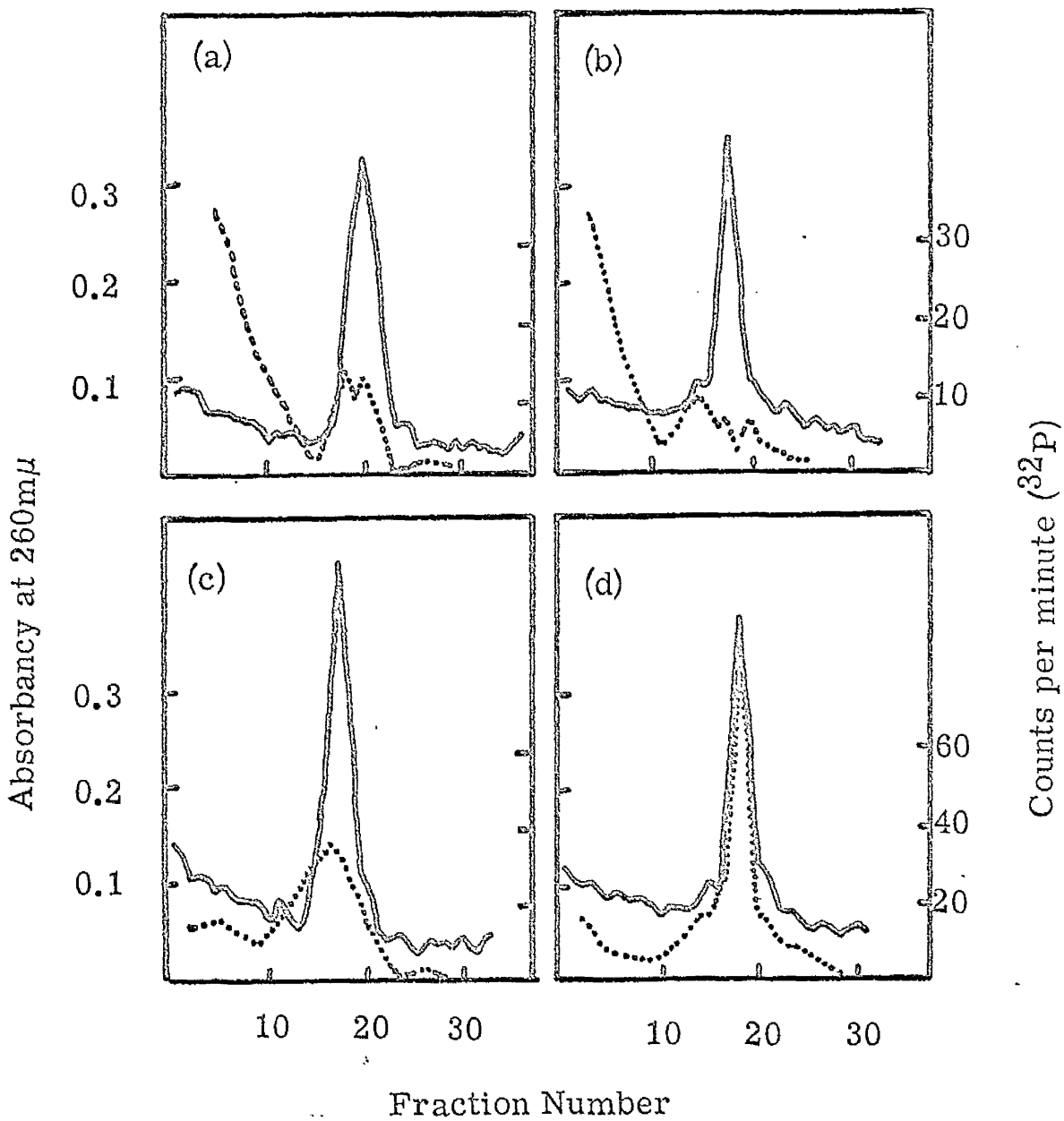
(c) Cell DNA with virus sRNA:

(d) Virus DNA with virus sRNA

——— Absorbancy at 260 mμ

– – – – Radioactivity

FIGURE 56


(a)
(b)
(c)
(d)
0.3
0.2
0.1
30
20
10
60
40
20
10
20
30
Absorbancy at 260mμ
Counts per minute (^{32}P)
Fraction Number

(c) Further DNA: sRNA Hybridisation Experiments.

sRNA extensively purified by the above two procedures (DEAE cellulose and MAK columns) was used in a DNA:sRNA hybridisation experiment. 5 µg. of control or infected cell sRNA was mixed with 50 µg. of heat-denatured BHK21 (C13) cell DNA or 50 µg. of heat-denatured Herpes Simplex virus DNA. Hybridisation was carried out exactly as described in the previous experiment, except that the radioactivity measurements were made after RNase treatment only. The four analyses are presented in Fig. 56.

Qualitatively, they indicate (i) that Herpes Simplex virus DNA hybridises with infected cell sRNA (Fig. 56d) but not with uninfected cell sRNA (Fig. 56b) and (ii) that cell DNA hybridises with both infected and uninfected cell sRNA (Fig. 56c,a). Quantitatively the results (Fig. 56d) show that approximately 1.2% of the viral DNA specifically hybridises with the infected cell sRNA. This represents a length of DNA sufficient to code for 10 - 20 molecular species of tRNA.

It is possible, under the above conditions, that the "hybridised" radioactivity ^{32}P observed in incubation mixtures containing infected cell sRNA was, in fact, viral DNA produced during the infectious cycle and sheared

F I G U R E 57.

(a) The experimental design of an assessment of the kinetics of production of sRNA after Herpes Simplex virus infection of BHK21 (C13) cells.

(b) The synthesis of sRNA in BHK21 (C13) cells after infection with Herpes Simplex virus.

The sRNA was exhaustively purified on MAK columns.

------ Uninfected cells (^{14}C) specific activity

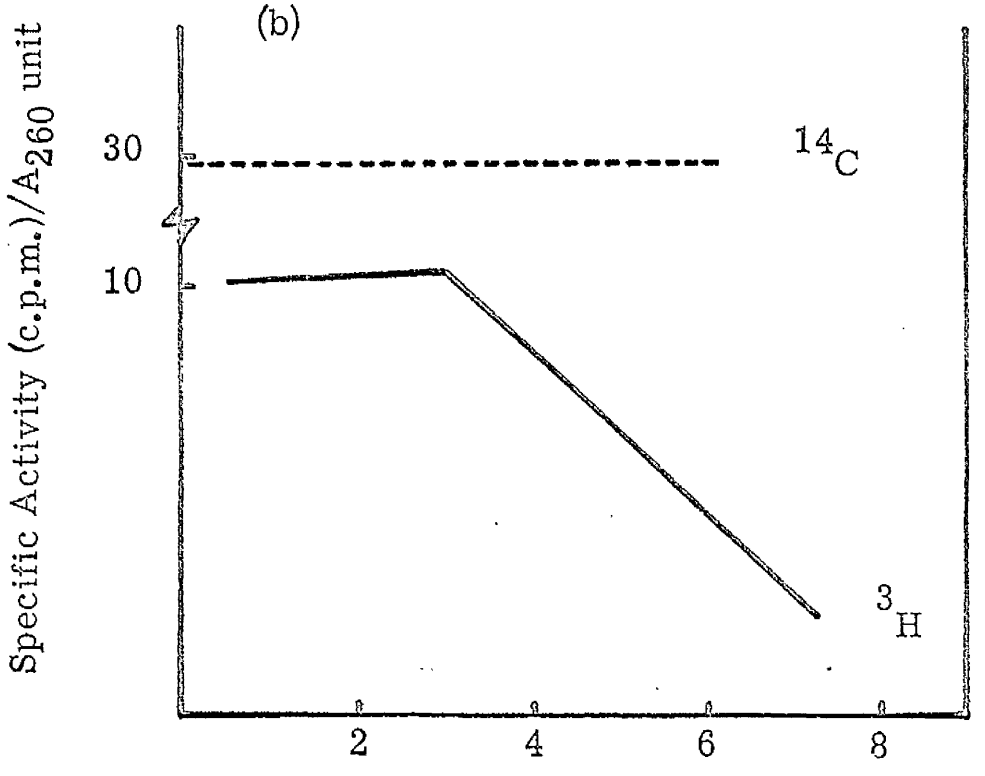
_____ Infected cell (^{3}H) specific activity

FIGURE 57

(a)

CONTROL (^{14}C)		INFECTED (^{3}H)	
pulse time (hr.)	sample	pulse time (hr.)	sample
1 - 3	C1	0 - 2	I1
1 - 8	C2	2 - 4	I3
		4 - 6	I4
		6 - 8	I5
		1 - 8	I2

(b)



to the size of sRNA on extraction. This DNA, of course, would sediment through a gradient of CsCl to the same position as herpes DNA. I tested for its presence, therefore, by centrifuging both the control and infected cell sRNA samples in CsCl and treating the fractions as above. No such contamination of the sRNA was detectable by absorbancy or radioactivity measurements.

16. KINETICS OF PRODUCTION OF sRNA IN HERPES SIMPLEX VIRUS-INFECTED CELLS.

(a) Since ^{32}P is a non-specific and short-lived precursor of RNA it was decided to use labelled uridine in subsequent experiments. The following was designed to investigate the time course of production of sRNA in herpes-infected cells, and to provide sRNA pulsed for short times after infection as well as 7 hour-labelled material for hybridisation purposes. The design is shown in Fig. 57a.

All sRNA was purified by the method of Hoagland (Hoagland [344]) and further fractionated on MAK columns (Methods Sect. 2(b)). The specific activities of sRNA fractions as a function of time after infection are detailed in Fig. 57b. The material was assayed for radioactivity by precipitation on teflon-coated glass fibre membranes (Methods Sect. 6(h)(ii)).

F I G U R E 58.

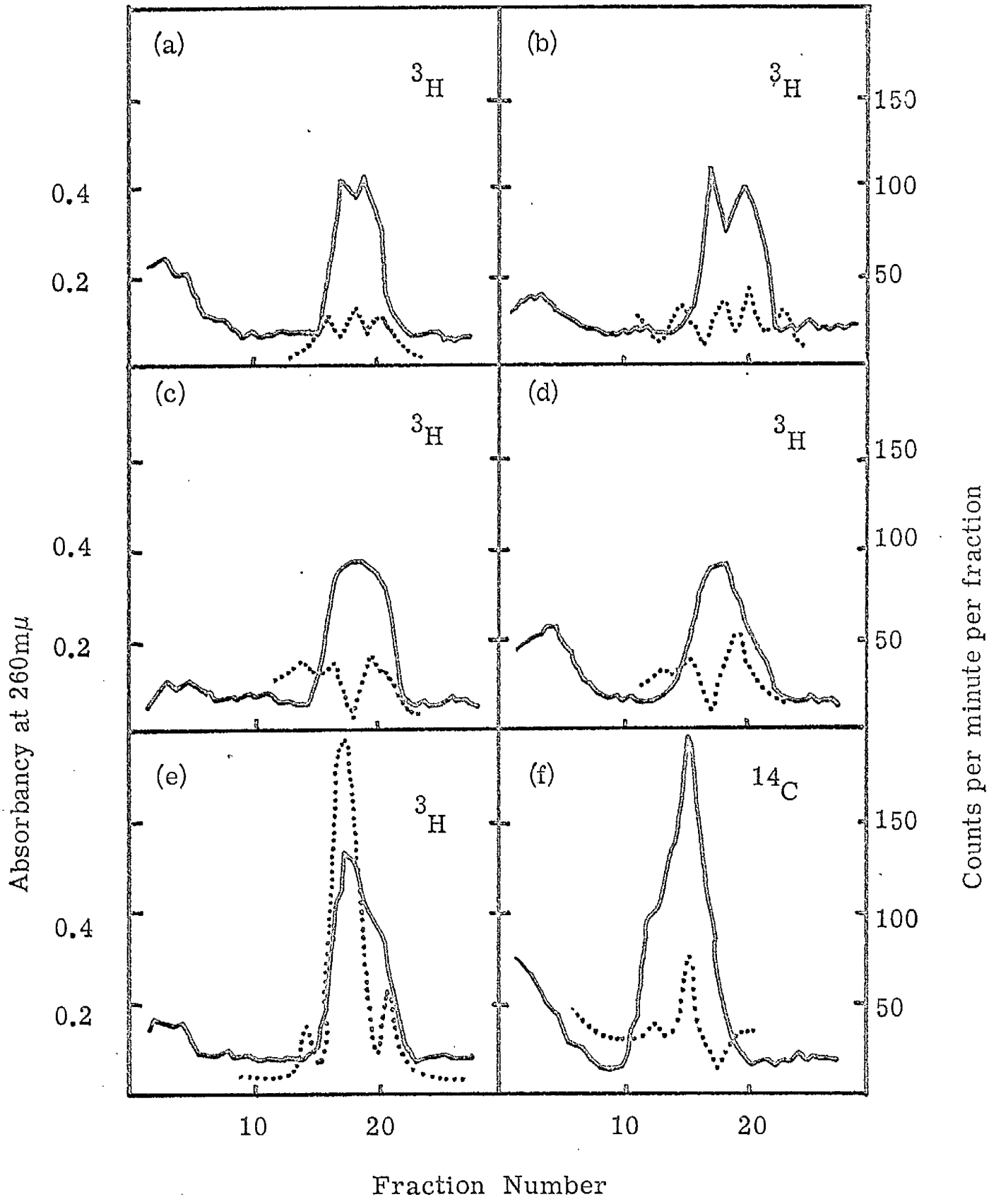
CsCl equilibrium centrifugation of hybridisation mixtures of sRNA and DNA from infected and uninfected BHK21 (C13) cells.

BHK21 (C13) cell and Herpes Simplex virus DNAs were hybridised with sRNA isolated from infected and uninfected cells at several intervals after infection. The formed hybrids were fractionated in CsCl gradients. Each fraction was tested for RNase sensitivity. (a),(b),(c),(d) and (e) represent respectively 11,13,14,15 and 12 sRNAs with an equal mixture of host and viral DNAs. (f) represents C2 sRNA with a 3 to 1 mixture of host and viral DNA.

____ Absorbancy at 260mμ

____ Radioactivity

FIGURE 58


(a)
^{3}H
(b)
^{3}H
(c)
^{3}H
(d)
^{3}H
(e)
^{3}H
(f)
^{14}C
0.4
0.2
150
100
50
10
20
Absorbancy at 260mμ
Counts per minute per fraction
Fraction Number

This Figure shows that sRNA production in herpes-infected BHK21 (C13) cells begins to decline only after four hours of the infectious cycle, but during the next four hours it falls by 80%.

Hybridisation experiments were now carried out involving the DNA's of the host and virus and the sRNA samples from the Kinetic experiment above (with the exception of sample C1). Host and virus DNA (50 ug. of each, heat denatured) were mixed with 5 µg. of the sRNA from each of the five infected cell samples I1, I2, I3, I4, I5 and the 5 µg. of the control sample C2. was similarly mixed with 50 µg. of heated virus DNA and 150 µg. of host DNA. All six mixtures were incubated, analysed on CsCl gradients and fractionated as described previously (Results Sect. 15c): the results are given in Fig. 58. It may be noted that in none of the tubes has separation between the host and the virus DNA been complete.

An examination of the patterns shown in Fig. 58e and f which give the results for hybridisation using I2 and C2 sRNA samples respectively (both labelled for 7 hours) confirms results obtained using other sRNA samples (cf. Sect. 15c). The infected cell sRNA hybridises with virus DNA to a large extent and with host DNA (lighter)

to a much smaller degree (Fig. 58e), while control cell sRNA (Fig. 58f) hybridises only with host cell DNA.

Fig. 58 a,b,c and d are difficult to interpret. They involve sRNA pulsed for 2 hours at successive times after infection. Initially (Fig. 58a), little evidence of virus DNA - RNA association can be seen, but some host DNA - RNA hybridisation may be present. The last time sample of sRNA (Fig. 58d) shows two peaks of DNA-associated RNA, possibly corresponding to host and viral DNA hybridisation with the infected cell sRNA; the samples intermediate between these two are poorly defined.

(b) Examination of the stability of the DNA: sRNA hybrid to exhaustive RNase treatment.

Purified sRNA labelled with [^{3}H] uridine from the last experiment was used in the following test. sRNA sample I2, which had been exposed to precursor from 1 to 8 hours after infection with Herpes Simplex virus was incubated (1 ug.) with either 50 µg. of heat denatured Herpes Simplex virus DNA or 70 µg. of heat-denatured BHK21 (C13) DNA under standard hybridisation conditions (Methods Sect. 9(b)). In short, this experiment represents a repeat of part of experiment

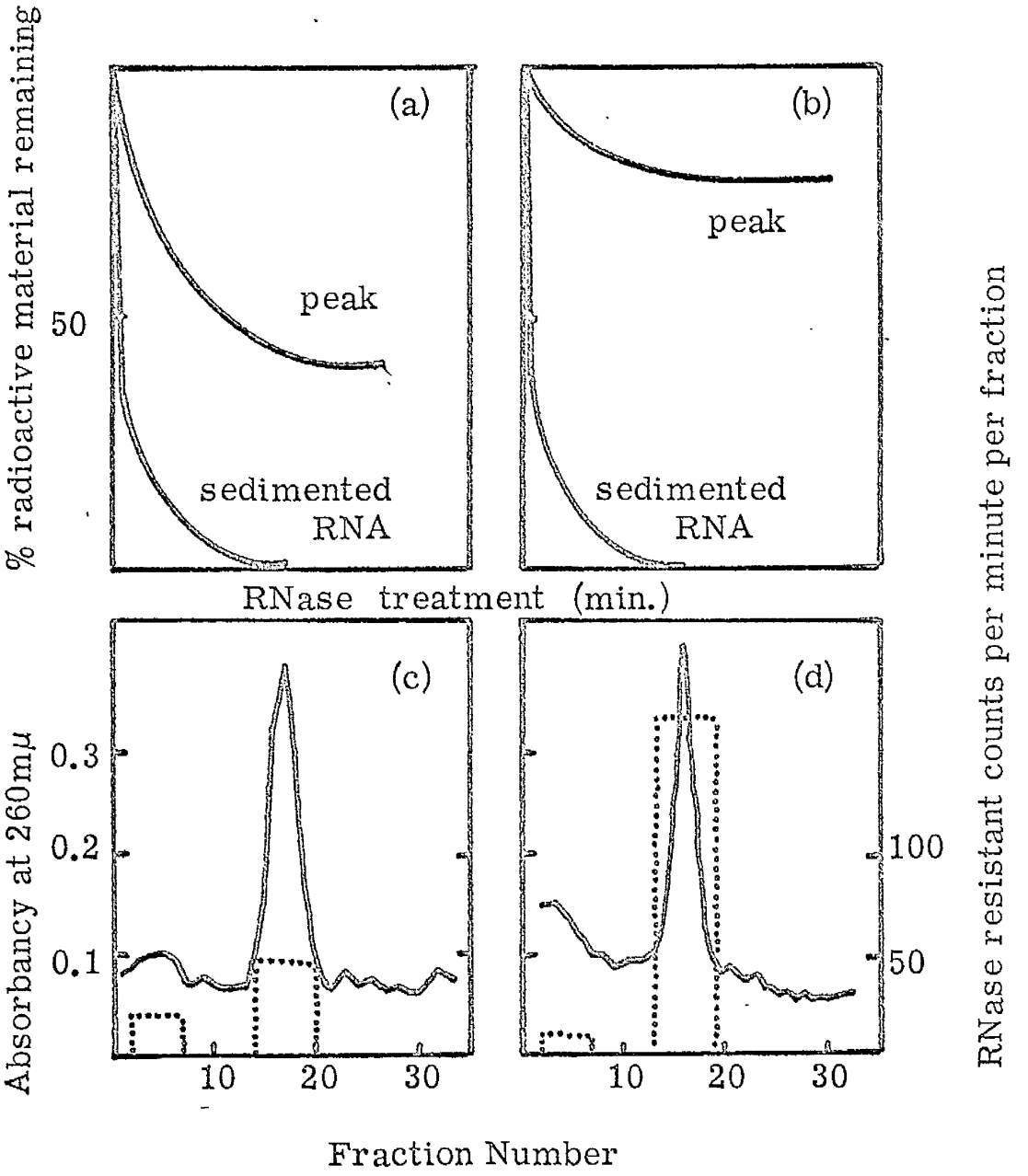
F I G U R E 59.

The Resistance of the DNA:sRNA hybrid to RNase treatment. BHK21 (C13) cell DNA and Herpes Simplex virus DNA were hybridised with sRNA from infected cells. The material used was sample T2 (see Fig.57a). Fractionation of the formed hybrids in gradients of CsCl gave rise to two components for each analysis: the peak tubes (containing hybrid) and the free sedimented RNA. (c - host DNA; d - virus DNA). The kinetics of RNase action on these four fractions are shown (a - host material; b - virus material).

——— Absorbancy at 260mμ

⬚⬚ RNase-resistant radioactivity (total).

FIGURE 59


% radioactive material remaining
50
(a)
peak
sedimented RNA
(b)
peak
sedimented RNA
RNase treatment (min.)
Absorbancy at 260mμ
0.3
0.2
0.1
(c)
10
20
30
(d)
10
20
30
100
50
RNase resistant counts per minute per fraction
Fraction Number

15c, except in the following sense: after CsCl density gradient fractionation of the two samples (Methods Sect. 8(b)), the sedimented RNA in both and the RNA associated with the DNA in both (see Fig. 59 c and d) were separately pooled, diluted to 0.2M CsCl and treated for up to 30 minutes with 5 µg./ml. of RNase A (Worthington, previously heated to 100° for 20 minutes).

Fig. 59 a and b relate the duration of RNase treatment to the percentage radioactivity remaining in the 5% trichloracetic acid-insoluble material for the four pooled fractions (Methods Sect. 6(h)(iii)). The observed values at completion of digestion were: sedimented RNA 0.9%, i.e. 20/2222 (Fig. 59a) and 0.7%, i.e. 12/1714 (Fig. 59b); absorbancy peak 43% i.e. 47/109(a) and 77%, i.e. 168/218(b).

These results confirm and extend the findings on hybridisation between BHK21 (C13) cell and Herpes Simplex virus DNA and sRNA from infected cells, and gives more detailed information on the nature of the hybrid association.

17. PHYSICAL MEASUREMENTS ON sRNA.

(a) Ultra-violet spectra.

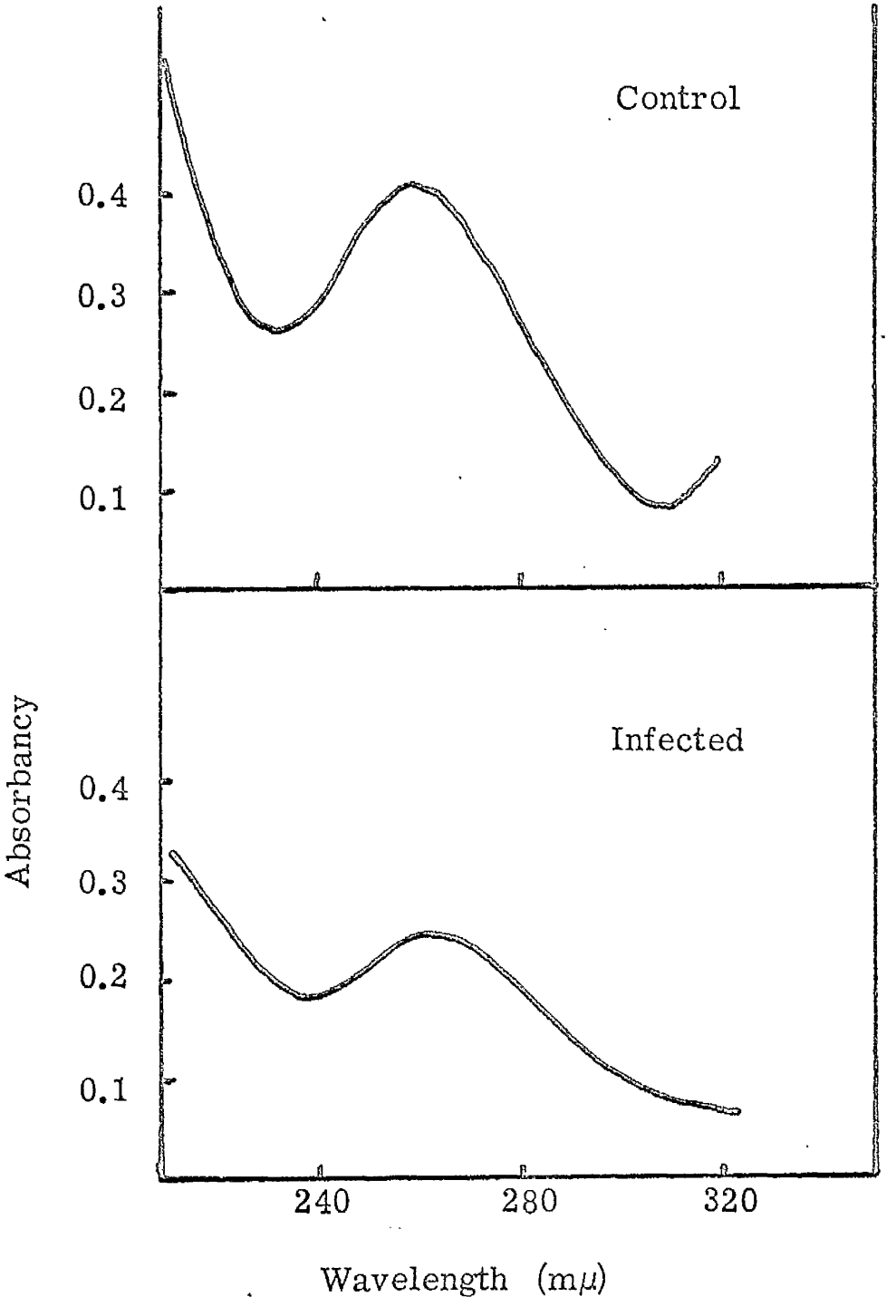
Measurements of the absorbancy of solutions of

F I G U R E 60.

Ultraviolet Absorption spectra of sRNA preparations.

The spectra were measured at pH7.

FIGURE 60


Control
Infected
0.4
0.3
0.2
0.1
0.4
0.3
0.2
0.1
Absorbancy
240
280
320
Wavelength (mμ)

BHK21 (C13) cell sRNA and sRNA from cells (both isolated by the standard technique (Results Sect. 15b) 7 hours after infection with Herpes Simplex virus were carried out from 320 to 200 mμ.

Both sRNA samples were dissolved in 0.01M Tris-HCl buffer pH7.0 at 10 μg./ml. and measurements taken using the Beckmann DB recording spectrophotometer and a 3.5 ml quartz cuvette.

While the spectra (Fig. 60) are characteristic of nucleic acid spectra at this pH, no point of difference between the two sRNA samples can be detected. If (i) the tRNA present in BHK21 (C13) cells has a base composition which is not similar to that of tRNA elaborated after Herpes Simplex virus infection and (ii) if this latter tRNA is present to a significant extent relative to the former, then, only under these conditions, would differences in absorption spectra between the two samples measured above have been observed.

(b) Sedimentation coefficient measurements.

sRNA's from BHK21 (C13) cells, uninfected and infected with Herpes Simplex virus (from Experiment 15c) were diluted to 40 μg./ml. with 0.14M NaCl at pH 7.0 and centrifuged at 59,780 r.p.m. in the Spinco Model E

F I G U R E 61.

Calculation of the Sedimentation

Coefficients of sRNA preparations.

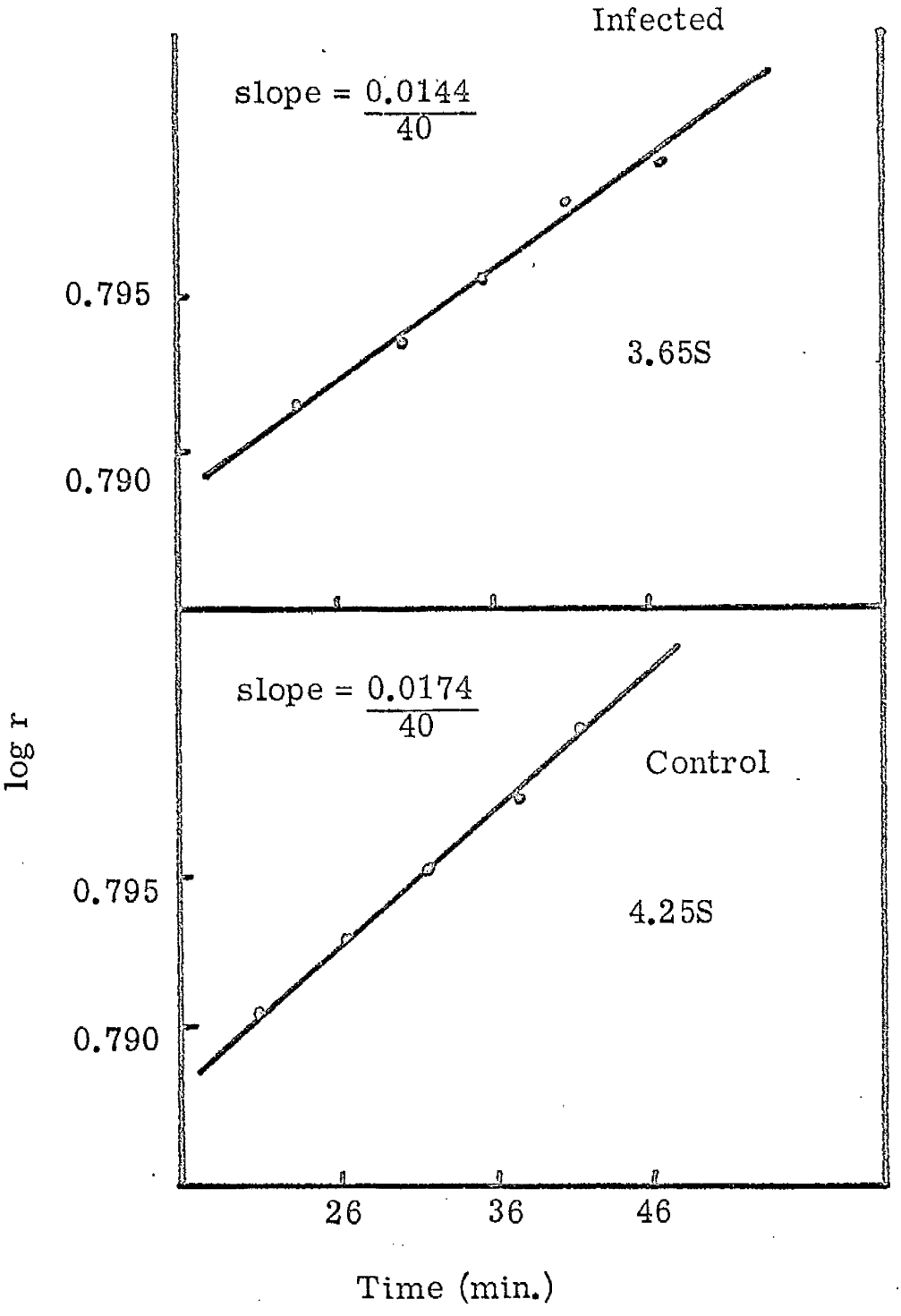
x = distance of the moving boundary of solute from the centre of rotation of the rotor.

w = rotor speed in radians per second.

Slope of the curve = $\frac{d\log x}{dT}$

$$S = \frac{1}{w^2} \times \frac{d\log x}{dT} \quad \text{sec}^{-1}$$

FIGURE 61


Infected
slope = 0.0144/40
0.795
0.790
3.65S
slope = 0.0174/40
Control
0.795
0.790
4.25S
log r
26
36
46
Time (min.)

analytical ultracentrifuge. Photographs of the moving boundary (using ultraviolet light) were taken at 4 minute intervals and the curves of this movement as a function of time for both sRNA's are shown (Fig. 61). S values were calculated using the method of Svedberg & Nicols (Svedberg & Nicols [352]), and these were 3.65 for the infected cell sRNA and 4.25 for uninfected cell sRNA.

The significance of these relatively small differences in S value between sRNA's before and after infection is not easy to assess, but the boundaries of sedimenting material observed in these analyses were consistently sharp. This indicates that very little material whose S value differed significantly from the above values was present in either sRNA sample, further confirming the molecular homogenicity of these preparations.

17.

(c) Melting temperature determinations.

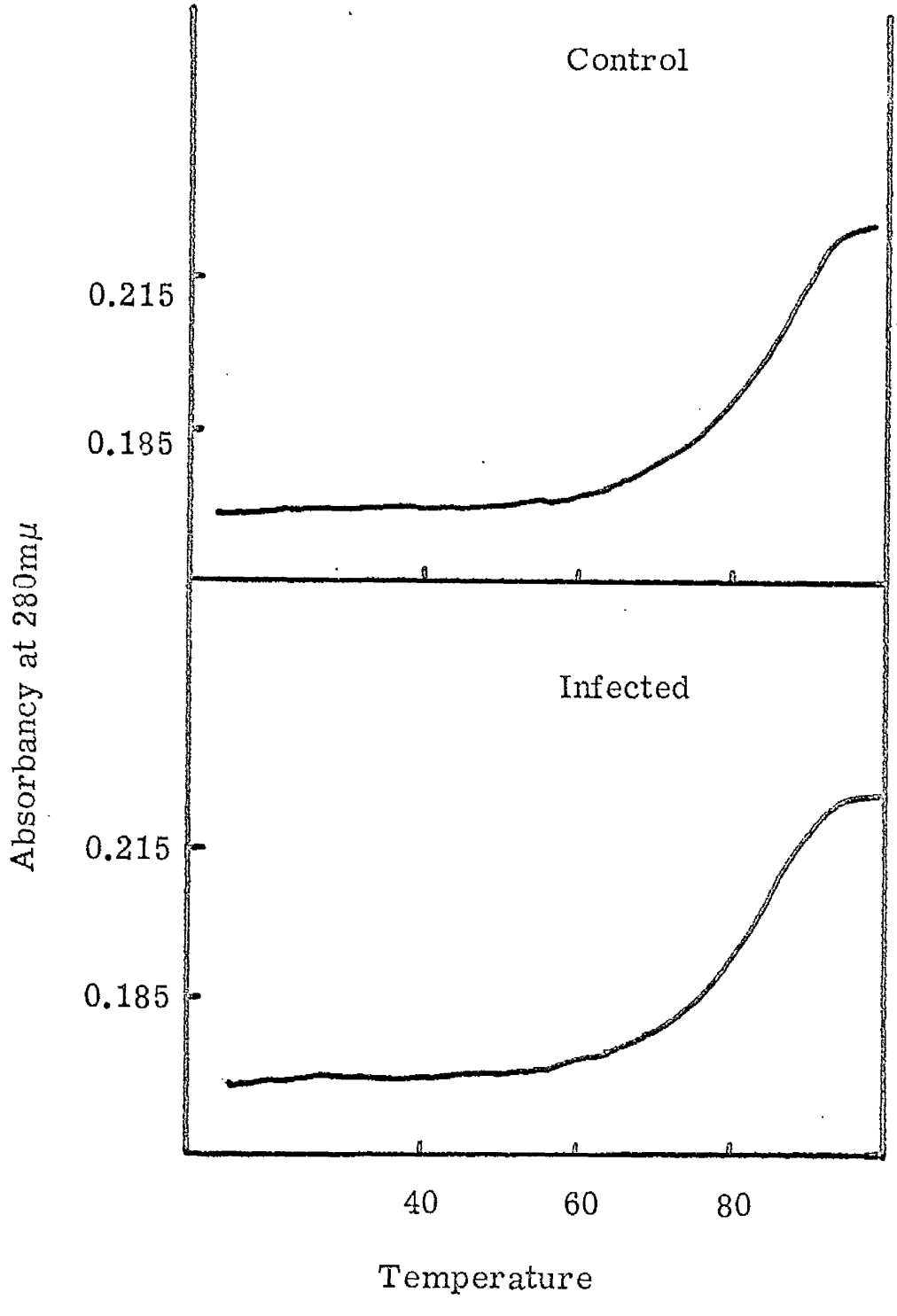
Two nucleic acid preparations which contain tertiary structure and which have different base compositions will exhibit characteristically different absorbancy increase patterns when heated in solution.

F I G U R E 62.

The Effect of temperature on the Absorbancy at 260 mμ of sRNA preparations.

The 'melting temperature' is taken as the mid-point of the curve of hyperchromicity.

FIGURE 62


Control
0.215
0.185
Infected
0.215
0.185
Absorbancy at 280mμ
40
60
80
Temperature

If Herpes Simplex virus infected cell sRNA contains two sRNA fractions (one host specified, one virus specified), then by using such a technique it may be possible to distinguish these two components.

Separately, BHK21 (C13) cell sRNA and sRNA from Herpes Simplex virus-infected cells were prepared by the standard technique (Results Sect. 15b) and dissolved (to 20 µg./ml.) in 0.14M NaCl containing 0.05M $MgCl_2$. Each was then pipetted into a 3.5ml quartz cuvette (1 cm. light path) and slowly heated to 100°C using the Unicam SP 800 spectrophotometer. The measurements of hyperchromicity, made at 280mµ, are shown as a function of temperature in Fig. 62. The "Tm" for each sRNA preparation was measured at the mid-points of the above curves as shown. These were 82° for control and 81° for infected material. These values do not represent two substantially different sRNA preparations, nor is there evidence (Fig. 62) that either preparation contains more than one component.

sRNA's generally have a high G plus C content: this base pair exhibits, during the helix-coil transition, a more marked hyperchromicity at 280mµ than at 260mµ, hence the use of this wave length in the analysis.

18. AN ATTEMPT TO HYBRIDISE INFECTED CELL sRNA WITH NATIVE BHK21 (C13) DNA AND A BACTERIAL DNA.

It has been established that sRNA purified from herpes-infected BHK21 (C13) cells forms RNase-resistant hybrids with both host and viral DNA. This 'hybridisation' may be the result of a non-specific binding phenomenon between this RNA and DNA: hence DNA from Escherichia coli B was tested in the standard hybridisation system.

Consideration of the possible function of the small, virus-coded RNA suggested the possibility that this RNA may not have an amino-acid transfer function as suggested, but might be involved in binding to the host DNA in vivo. Therefore, concomitantly with the experiment involving bacterial DNA, binding between infected cell sRNA and double-stranded DNA from BHK21 (C13) cells was investigated under the standard conditions and also at room temperature. The sRNA used was the 'I2' sample from Experiment 16a.

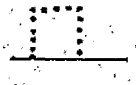
50 µg. of BHK21 (C13) cell DNA (unheated) was mixed under standard ionic conditions (Methods Sect. 9(b)) with 5 µg. of 'I2' sRNA and incubated at 20° for 2 hours. A second identical tube and a third containing 50 µg. of heat-denatured Escherichia coli B DNA, 5 µg. of 'I2' sRNA and the standard ionic complement were incubated under the standard conditions.

F I G U R E 63.

Attempted hybridisation of infected coli sRNA with DNA of Escherichia coli and with native DHK21 (C13) coli DNA.

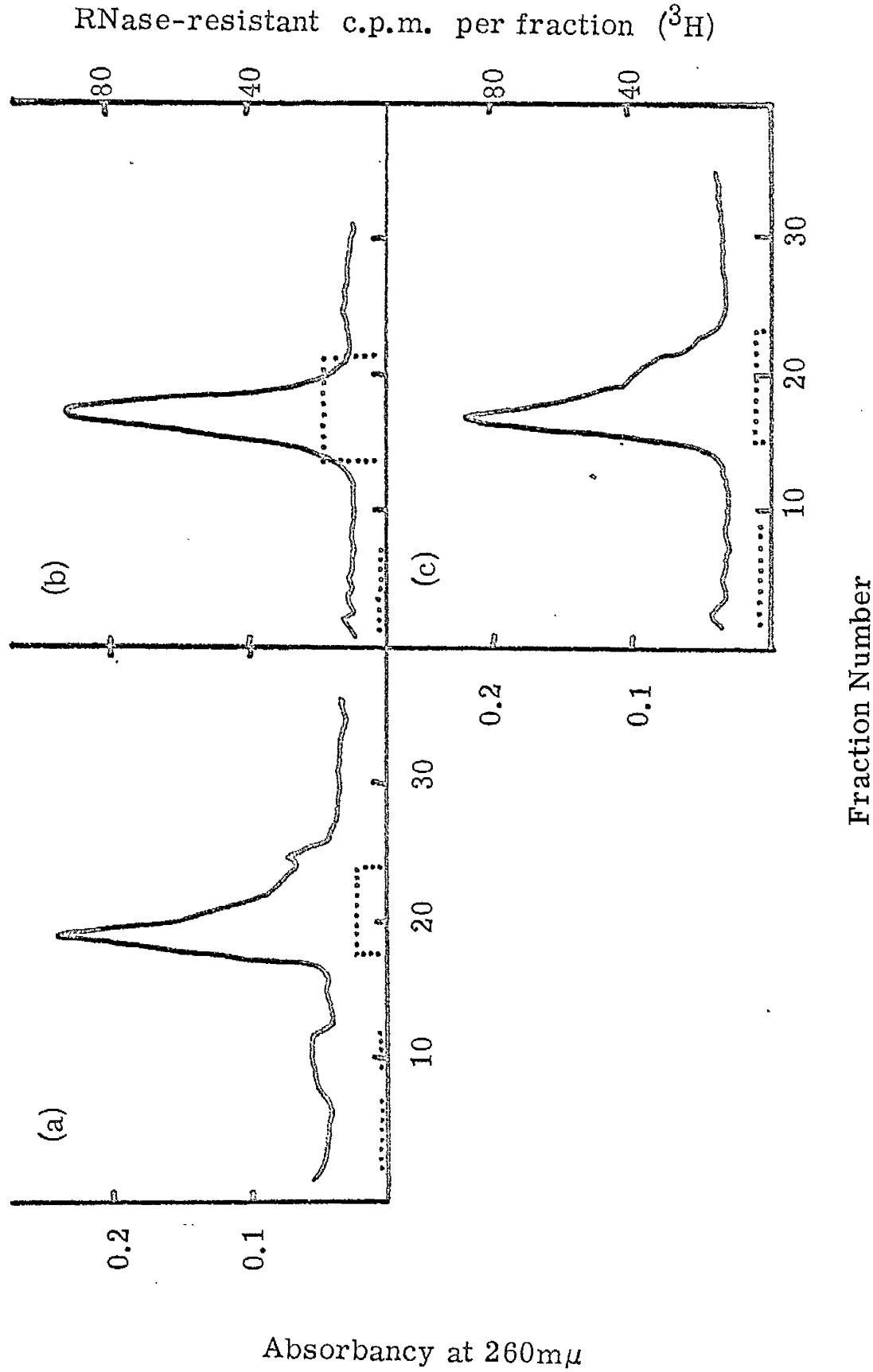
The hybridisation mixtures were as follows: (a) native DHK21 (C13) coli DNA with sRNA, (b) Escherichia coli DNA with sRNA and (c) native DHK21 (C13) coli DNA with sRNA but incubated at 20°. The sRNA used was sample E2 from Fig.57a. DNA associated and sedimented RNA was treated with RNase after fractionation in CsCl gradients.

——— Absorbancy at 260mμ



RNase-resistant radioactivity (total)

FIGURE 63


RNase-resistant c.p.m. per fraction (^{3}H)
80
40
(b)
(c)
(a)
0.2
0.1
10
20
30
Absorbancy at 260mμ
Fraction Number

All three mixtures were fractionated on CsCl density gradients. "DNA-associated" sRNA and "sedimented" sRNA fractions in all tubes were pooled, treated with RNase over 30 minutes and assayed for radioactivity (Results Sect. 16b).

The results (Fig. 63) indicate that no significant amount of RNase-resistant DNA:sRNA complex has formed with double-stranded BHK21 (C13) cell DNA either under standard incubation conditions (Fig. 63a) or at room temperature (Fig. 63c). On the other hand, heat-denatured Escherichia coli DNA forms a significant, if small amount of specific hybrid (Fig. 63b). This may be biologically significant: it may, alternatively, merely represent the background level of such an analysis. Quantitatively, these results may be compared with those in Fig. 59, which represent an identical test using the same sRNA fraction.

19. FORMATION OF AMINOACYL tRNA.

The hybridisation experiments have given evidence for the presence in sRNA preparations of an RNA fraction which is derived from Herpes Simplex DNA transcription: theoretical and physical considerations suggest that this RNA may be tRNA. If this is so, then the sRNA should be capable of accepting amino acids; the sRNA's

were tested in the following way:

Unlabelled sRNA was prepared from BHK21 (C13) cells and from Herpes Simplex virus infected BHK21 (C13) cells 8 hours after infection (Methods Sect. 5(c)). Since at least some part of the RNA might be expected to be present as aminoacyl tRNA, it is important to ensure removal of all amino acid before embarking on a "loading" experiment. Both sRNA preparations from above were therefore precipitated with ethanol and incubated with Tris-HCl buffer using the standard method for removal of bound amino acid (Methods Sect. 11(a)).

19. PREPARATION OF Aligase AND USE OF CRUDE PREPARATION.

(a) The enzymes which *in vivo* catalyse the formation of aminoacyl tRNAs are called aminoacyl tRNA synthetases (Aligases): these enzymes appear to be amino acid-specific (Brown [72]).

ARSase was prepared from BHK21 (C13) cells and cells infected with Herpes Virus for 8 hours (Methods Sect. 10(b)). The preparations were used immediately in this case.

Four tubes were set up, and all contained the following: one µmole ATP, 10 µmoles KCl, 2 µmoles $MgCl_2$, 10 µmoles Tris-HCl buffer pH 7.6 and 40 µmoles of a

TABLE 13.

Analysis of ligase activity in soluble extracts of uninfected and Herpes Simplex virus infected BHK21 (C13) cells, and the use of preparations fractionated on DEAE-cellulose.

[^{14}C] algal protein hydrolysate was employed as source of labelled amino acids.

TABLE 13

(a) Crude ligase

Material from	+RNA c.p.m.	-RNA c.p.m.
MK 21 (C13) cells	87	46
Herpes-infected MK 21 (C13)cells	367	192

(b) DEAE-cellulose-treated ligase

Material from	+RNA c.p.m.	-RNA c.p.m.
MK 21 (C13)cells	216	55
Herpes-infected MK 21 (C13)cells	405	135

$[^{14}C]$ labelled algal protein hydolysate in 0.2 ml. In addition, two of the tubes contained 0.05 ml. of the infected cell ligase preparation (IC, IT); one tube (IT) also contained 100 ug. of infected cell sRNA (prepared as above). The other two tubes served as controls (CC, CT) and contained the corresponding uninfected cell material.

All four tubes were incubated at 37°. After 30 minutes, duplicate 0.05 ml. portions were removed from each tube, diluted to 1 ml. with 400 µg. Bovine serum albumin and precipitated and washed with cold 10% w/v trichlor acetic acid. The pellet was dissolved in 0.2M NH_4OH and counted in the Nuclear Chicago gas flow counter using a thin end-window.

The results (Table 13a) indicate that, while the sRNA added to the incubation mixtures has undoubtedly bound some $[^{14}C]$ amino acid, the enzyme preparations must themselves have contained substantial amounts of sRNA. This would be a natural consequence of the method used in enzyme preparation.

(b) Use of partially purified ligase.

Removal of endogenous sRNA from the ligase preparations was effected by chromatography on columns

of DEAE-cellulose. This method is simple but time consuming, and yields a dilute enzyme preparation (Methods Sect. 10(a)).

The above "loading" experiment was duplicated using as enzyme the DEAE-cellulose product. This entails omitting KCl from the standard assay mixture, since the column eluate is 0.3M with respect to KCl. The results (Table 13b) indicate that sRNA has indeed been removed from both infected and control enzyme preparations, but not entirely.

Similar amounts of total protein were used in the last two experiments and it can be seen from Table 13 that some removal of non-enzyme protein results from the column procedure. The infected cell preparations are the more active.

This procedure was used in several subsequent analyses.

(c) Isolation of aminoacyl tRNA's.

The aminoacyl tRNA products of the last experiment (19b) derived from infected cell material (IT, IC) were isolated by extracting the incubated mixtures with an equal volume of 90% phenol, precipitating the aqueous residues with 67% ethanol and 2% sodium acetate and

washing the precipitates with ethanol exhaustively. These were dissolved in 1 ml. of 0.01M Tris-HCl buffer, pH 7.6 and 0.05 ml. duplicates were measured for radioactivity using the above procedure (Experiment 19a). The results showed that the aminoacyl tRNA product could be isolated intact, that the enzyme preparation (fractionated on DEAE-cellulose) contributed 35% of the total tRNA and that the specific activity of tRNA from the tube with purified sRNA added was higher than in the tube with enzyme preparation only. This suggests that the enzyme-introduced tRNA was already "loaded" with some amino acid.

(d) Stability of ligase and aminoacyl tRNAs.

A DEAE-purified ligase preparation from BHK21 (C13) cells was stored at -20° for seven days. At the end of this period, no enzyme activity remained in the preparation. A diminished activity was noted using a similar preparation, stored at -70°. Therefore ligase preparations were not routinely stored before use, but were used immediately after fractionation at 4°.

The aminoacyl tRNA preparation isolated in the last experiment was stored at 4° in 0.01M Tris HCl buffer pH 7.6: after four days, 55% of the bound amino

TABLE 14.

The Genetic Code.

The data are derived from the work of Nirenberg *et al.*[365] and Matthaei[386].

TABLE 14

Nucleotides					
1.	2.				3.
	U	C	A	G	
U	Phenylalanine Phenylalanine Leucine Leucine	Serine Serine Serine Serine	Tyrosine Tyrosine Leucine Leucine	Cysteine Cysteine Tryptophane	U C A G
C	Leucine Leucine Leucine Leucine	Proline Proline Proline Proline	Histidine Histidine Glutamine Glutamine	Arginine Arginine Arginine Arginine	U C A G
A	Isoleucine Alanine? Methionine Methionine	Threonine Threonine Threonine Threonine	Asparagine Asparagine Lysine Lysine	Ser./Ileu. Ser./Ileu. Arginine Arginine	U C A G
G	Valine Valine Valine Valine	Alanine Alanine Alanine Alanine	Aspartate Aspartate Glutamate Glutamate	Glycine Glycine Glycine Glycine	U C A G

acid had been removed. The literature (Ishida & Muira [358]) confirms this finding, and routinely, aminoacyl tRNA preparations have therefore been stored at -70° in 0.1M sodium acetate buffer, pH 5.5.

20. tRNA FORMATION USING A SINGLE LABELLED AMINO ACID.

It has been established that sRNA preparations from BHK21 (C13) cells and from herpes-infected BHK21 (C13) cells accept amino acids *in vitro*.

The original theoretical considerations (Results Sect.15) based on nearest neighbour frequency analysis are relevant at this point. BHK21 (C13) cell DNA has a CpG doublet frequency only 9% that of herpes DNA. Therefore, if Herpes Simplex virus is required to manufacture (on infection of a BHK21 (C13) cell) tRNA molecules in order to adjust the existing tRNA population to meet its particular requirements, then it must synthesise tRNAs corresponding to amino acids whose assigned triplet codons contain the doublet CpG. Table 14 lists all of the known codons (Nirenberg *et.al.* [365]), and it can be seen that the relevant amino acids are arginine (especially), proline, serine, threonine and alanine.

Several tRNAs are known to exist for many amino

acids, and these have been separated analytically on MAK columns by several groups (e.g. Yamane et.al. [240]). If Herpes Simplex virus produces new tRNA molecules, then these may be (but are not necessarily) separable from host tRNA's on these columns. In order to test this possibility, aminoacyl tRNA's were loaded enzymically with the above amino acids and examined on MAK columns.

(a) Synthesis of Arginyl tRNA.

The sRNA preparations from control and 8 hour-infected BHK21 (C13) cells were carried out as previously, as was the isolation and DEAE-cellulose fractionation of the ARSase preparations from control and infected cells (Results Sect. 19b). In addition, however, the ARSases were dialysed against 0.02M Tris HCl pH 7.5 for 60 minutes prior to use to remove endogenous free amino acid from the preparation.

The incubation mixtures for aminoacyl tRNA synthesis were similar to those described in Experiment 19a, but GTP was added to one umole/ml. and nineteen unlabelled amino acids were included (Methods Sect.11(b)), all at 100 mμmoles/ml. final concentration.

Two such mixtures were made up. To one was added 500 μg. "unloaded" BHK21 (C13) cell sRNA, 2.0 A_{280}

F I G U R E 64.

Fractionation on MAK columns of arginyl tRNA from Herpes Simplex virus-infected and uninfected BHK21 (C13) cells.

(a) control cell material

(b) infected cell material

(Ligase was purified on DEAE-cellulose).

Control peaks - 0.50M & 0.55M NaCl;

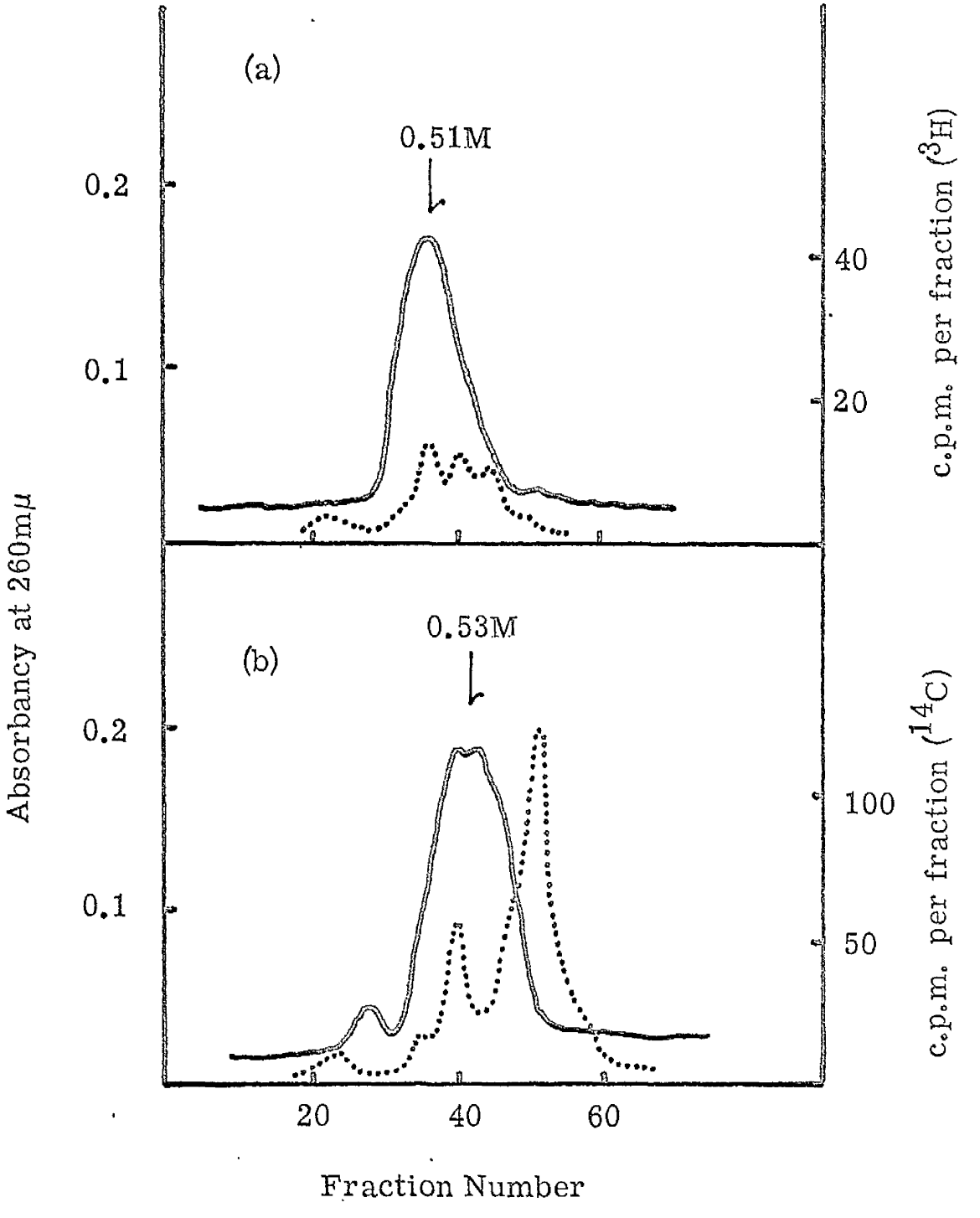
Infected peaks - 0.52M & 0.62M NaCl.

A linear gradient of NaCl was employed in elution.

_____ Absorbancy at 260 mμ

_ _ _ _ _ Radioactivity

FIGURE 64


(a)
0.51M
0.2
0.1
40
20
(b)
0.53M
0.2
0.1
100
50
20
40
60
Absorbancy at 260mμ
c.p.m. per fraction (^{3}H)
c.p.m. per fraction (^{14}C)
Fraction Number

units of control ligase preparation and 50 μC [^{3}H] arginine (50 mμmoles). The second mixture was similarly completed with infected cell sRNA and enzyme, but received 10 μC. (50 mμmoles) of [^{14}C] arginine.

Both samples were incubated for 17 minutes at 37°, (Ishida and Miura [358]) and the reaction stopped by the addition of 90% phenol. The deproteinised samples were washed thoroughly and each applied to a column of MAK at 0.15M NaCl (Methods Sect. 2(b)). The elution was via a linear gradient of NaCl and each 3 ml. fraction was assayed for absorbancy at 260 mμ in the Unicam SP 500 spectrophotometer. Radioactivity in one-third of each fraction was assayed by precipitation on cellulose nitrate membrane filters and scintillation counting (Methods Sect. 6(h)(ii)).

Patterns of absorbancy and radioactivity are given in Fig. 64. The absorbancy patterns are not significantly different; however the incorporation of labelled amino acid into control cell aminoacyl tRNA (Fig. 64a) has not taken place identically to that in the infected sample (Fig. 64b). In the control sample, the synthesised aminoacyl tRNA is eluted from MAK at 0.5M and 0.55M NaCl, while the corresponding infected cell aminoacyl tRNA's elute at 0.52M and 0.62M. Both control

peaks are probably present in the infected sample.

This result suggests strongly that the infected cells contain at least one arginyl tRNA which is not present in uninfected cells.

(b) Repeat of MAK fractionation on the infected cell arginyl tRNA.

As verification of the observed elution profiles, the infected cell arginyl tRNA was reabsorbed to a MAK column and eluted exactly as before.

The result was that although 70% of the applied sRNA adhered to the column, the aminoacyl-tRNA bond was broken by the treatment it received, and no radioactivity could be detected in the column eluate.

(c) Investigation of the enzymes involved in the formation of arginyl tRNA.

The last experiment suggested that after Herpes Simplex virus infection of BHK21 (C13) cells there appears at least one new species of arginyl tRNA. It is important now to investigate the possibility that the virus contains information to programme or induce the synthesis of a number of ligase molecules to allow these new tRNAs to be utilised. Against this hypothesis, however, is the fact (Doctor & Mudd [367]) that ligase

F I G U R E 6 5.

MAK column fractionation of arginyl tRNAs.

(a) control cell material

(b) infected cell material

(ligase was purified on DEAE-cellulose).

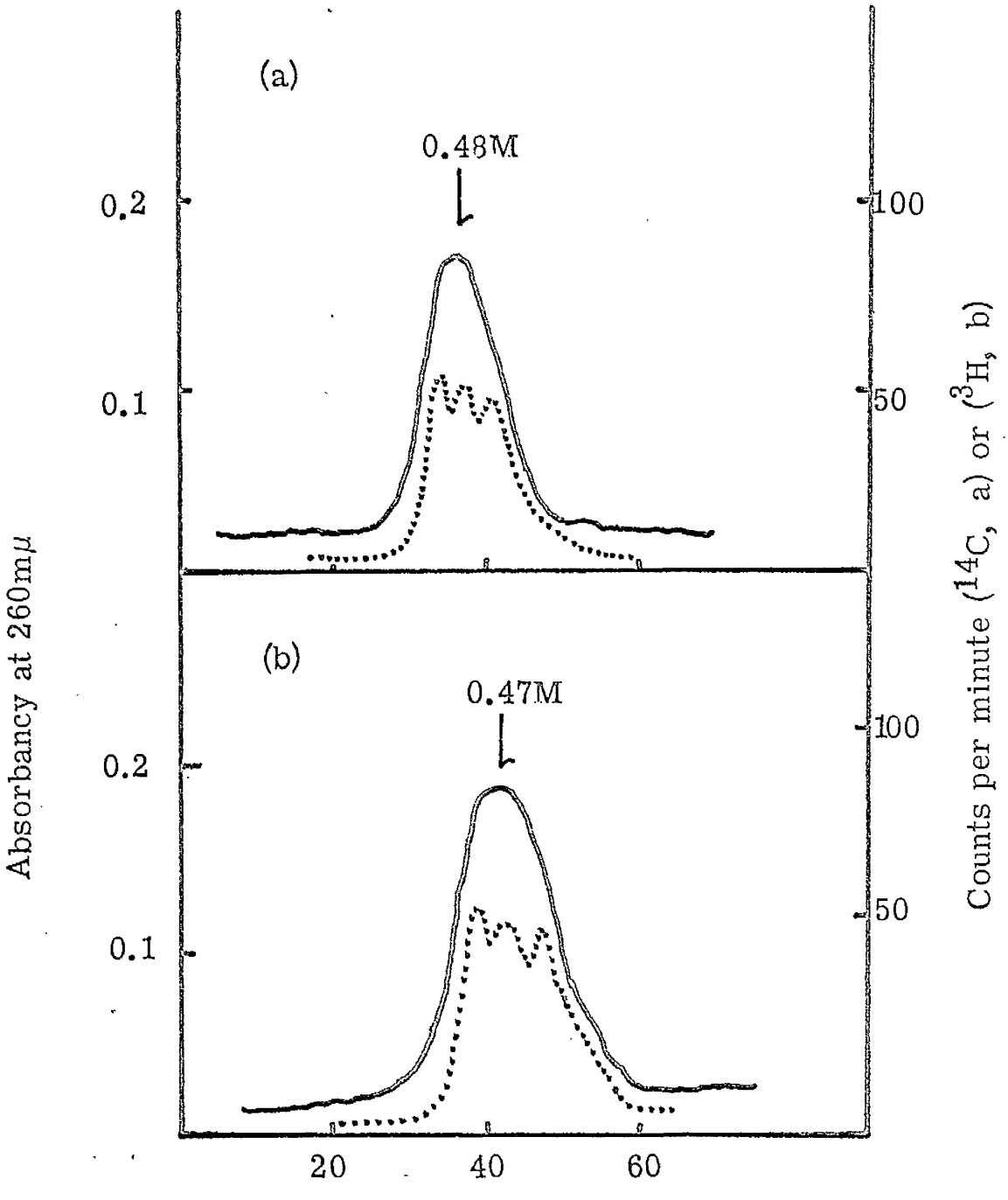
Both samples peak at 0.49M, 0.54M & 0.46M NaCl.

Material was eluted with a linear NaCl gradient

_____ Absorbancy at 260 mμ

\- - - - - - Radioactivity

FIGURE 65


(a)
0.48M
0.2
0.1
100
50
(b)
0.47M
0.2
0.1
100
50
20
40
60
Fraction Number
Absorbancy at 260mμ
Counts per minute (^{14}C, a) or (^{3}H, b)

molecules are not substantially specific within a population of tRNA molecules, themselves specific for an amino acid, and there would be no reason on this basis why the new tRNA's could not be "loaded" with the appropriate amino acids by the existing host enzymes.

Nevertheless, four reaction mixtures were set up to test the ability of control cell enzyme to form arginyl tRNA with infected cell tRNA and vice versa. The contents of each tube were exactly as described for the last experiment, except that tube one contained control cell enzyme and sRNA, tube two contained infected cell enzyme and sRNA, tube three contained infected cell enzyme and infected cell sRNA. The tubes with control and infected cell sRNA contained respectively $[^{14}C]$ arginine and $[^{3}H]$arginine. The incubation, isolation of product and MAK fractionation were carried out as in the last experiment.

Fig. 65 gives the results for the tubes containing control enzyme and control tRNA and infected cell enzyme and infected cell tRNA only. No significant difference can be observed between the profiles of the $[^{14}C]$ arginyl tRNA (control) and the $[^{3}H]$ arginyl tRNA (infected) in this analysis. Nor are these pictures exactly similar

F I G U R E 66.

MAK column fractionation of Arginyl tRNAs.

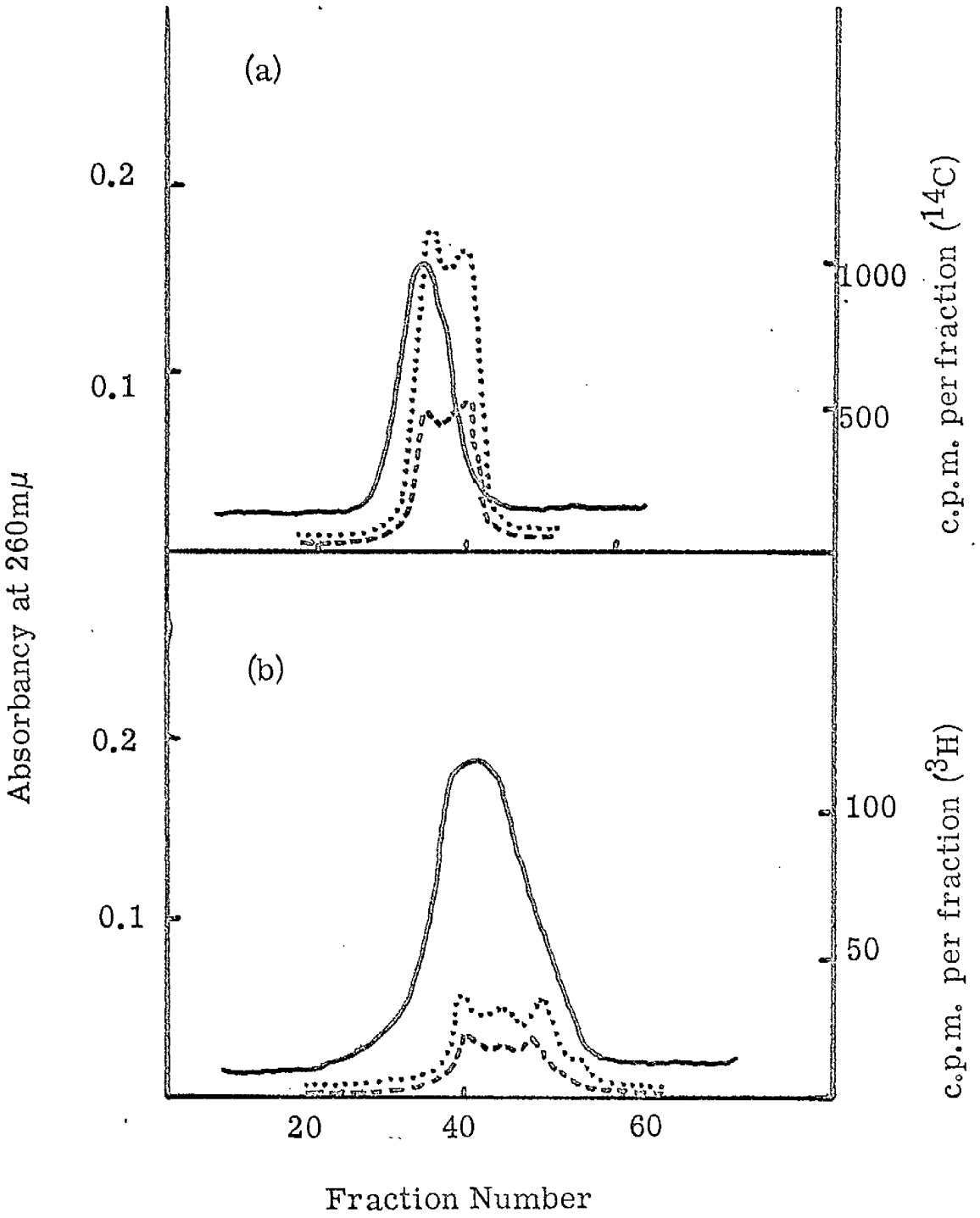
(a) Arginyl tRNA formed using BHK21 (C13) cell sRNA ligase (----) plus arginyl tRNA formed using Herpes Simplex virus-infected cell sRNA and ligase (.....).

(b) Arginyl tRNA formed using cell sRNA and infected cell ligase (----) plus arginyl tRNA formed using infected cell sRNA and control cell ligase (.....).

Elution with a linear gradient of NaCl gave patterns similar to previous analyses. Radioactivity eluted at 0.48M & 0.54M NaCl(a) and 0.46M, 0.49M & 0.54M NaCl (b). ARSases were fractionated on DEAE-cellulose.

———— Absorbancy at 260mμ

FIGURE 66


(a)
(b)
0.2
0.1
0.2
0.1
1000
500
100
50
20
40
60
Absorbancy at 260mμ
c.p.m. per fraction (^{14}C)
c.p.m. per fraction (^{3}H)
Fraction Number

to either the control or infected patterns obtained in Fig. 64. However, of the three peaks of radioactivity observed, two, at 0.49M and 0.54M correspond to the control peaks of Fig. 64. The third peak, confined apparently to Fig. 65 elutes at 0.46M, may exist as a shoulder on the control and infected profiles of Fig. 64. The difference between these two groups of analyses may reside in the more careful fractionation employed for Fig. 65. The loss of the aminoacyl tRNA peak at 0.62M (Fig. 64) in the infected cell sample (Fig. 65) may be due to the extreme instability of the relevant enzyme (cf. Section 19d): the enzyme preparations had, in this case, been stored at -70° for a week prior to use.

The remaining tubes were not analysed: in the light of the results obtained, their information would not have been meaningful.

(d) Ligase specificity reinvestigated.

The last experiment was repeated exactly, except that the ligase enzyme preparation, fractionated on DEAE cellulose, was used fresh in order to avoid criticism concerning the possible lability of certain ligase enzymes.

Fig. 66 shows the final result of the analysis,

and represents standard MAK column analyses. These were carried out on each sample separately, but the diagrams show these as two superimposed pairs. The superimposition was made on the basis of the NaCl eluting gradient employed. In this experiment the control enzyme and control sRNA and infected enzyme and infected sRNA tubes (Fig. 66a) were incubated with $[^{14}C]$ arginine in an attempt to quantitate the arginyl tRNA formation.

The remaining two tubes contained $[^{3}H]$ arginine (Fig. 66b). Fig. 66a reveals no qualitative difference between the control and herpes-infected cell systems on the basis of arginyl-tRNA formation. Both pictures are essentially replicates of the corresponding samples in the last experiment (Fig.65), but in this case the NaCl gradient is steeper and the resolution poorer.

The superimposed patterns of $[^{3}H]$ arginine incorporation into arginyl-tRNA in the two remaining samples (control sRNA, infected cell enzyme and infected cell sRNA, control enzyme) are given in Fig. 66b. Here the resolution is better than in Fig. 66a, but again no qualitative difference is notable.

In both sets of analyses, however, it is important to note that the specific activity of incorporation of arginine into infected cell sRNA is substantially greater than in the corresponding samples containing control

F I G U R E 67.

RAK column fractionation of Seryl tRNAs.

(a) Seryl tRNA from Herpes Simplex virus-infected cell sRNA and BHK21 (C13) cell ligase (.....) plus seryl tRNA from infected cell sRNA and ligase (- - - -).

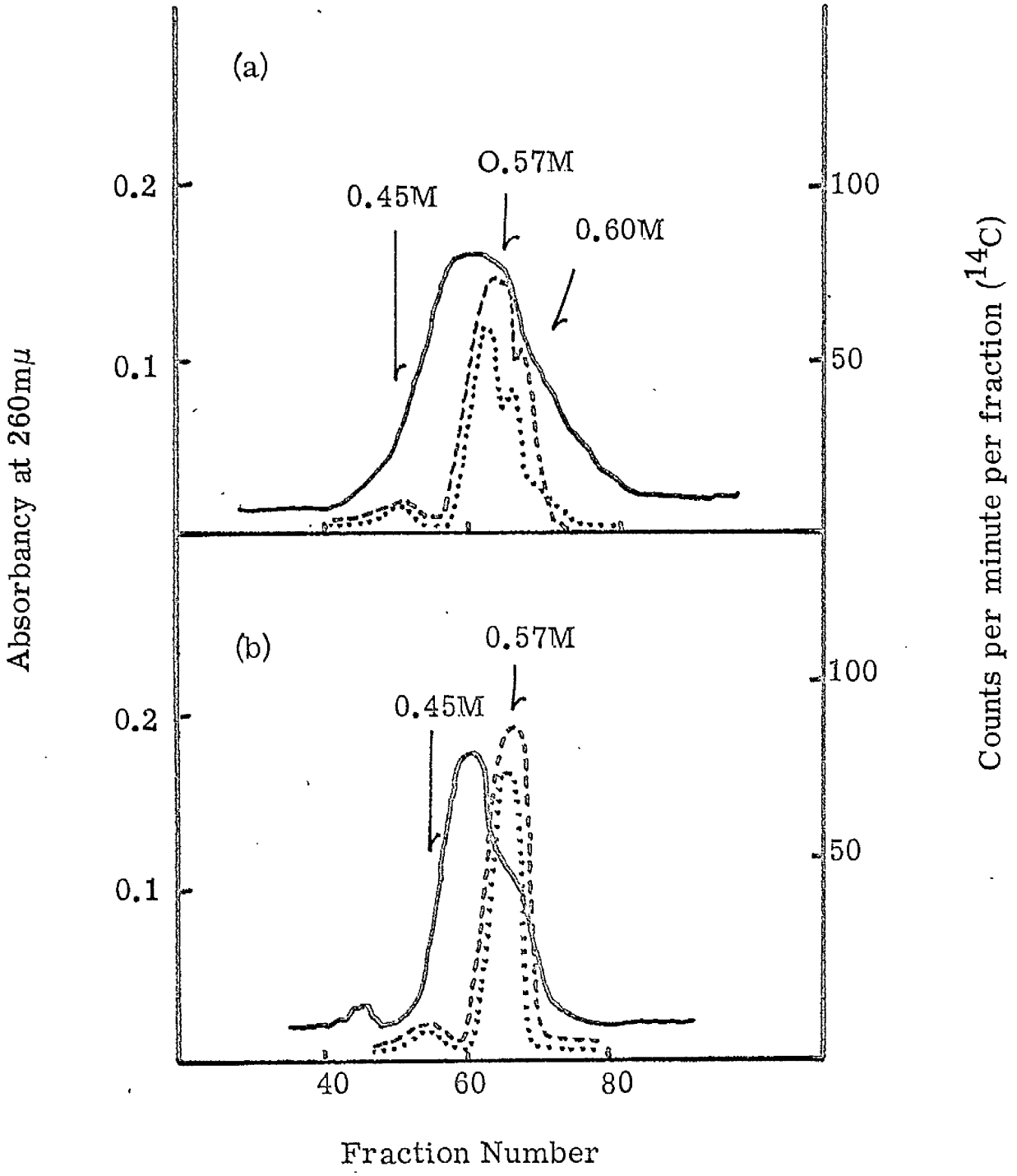
(b) Seryl tRNA from control cell sRNA and infected cell ligase (_ _ _ _) plus seryl tRNA from control cell sRNA and ligase (· · · · ·).

Elution with a linear gradient of NaCl gave rise to the peaks of radioactivity at the shown NaCl concentrations.

AARSases were purified on DEAE-cellulose.

———— Absorbancy at 260mμ

FIGURE 67


(a)
0.45M
0.57M
0.60M
0.2
0.1
100
50
(b)
0.57M
0.45M
0.2
0.1
100
50
40
60
80
Fraction Number
Absorbancy at 260mμ
Counts per minute per fraction (^{14}C)

cell sRNA. This is compatible with the existence of a new population of arginyl-tRNAs and also suggests that the control cell ligase enzymes are able to act on the "new" infected cell sRNA, forming arginyl-tRNAs.

21. FORMATION OF SERYL tRNA.

(a) Serine is an amino acid with a triplet codon containing the CpG doublet (cf. Results Sect. 20). Therefore this amino acid was incubated with sRNA and ligase (DEAE-cellulose fraction) from control (BHK21 (Cl3)) or Herpes Simplex virus infected cells. The design of the experiment, and the method of isolation and fractionation on NAK of the product seryl tRNA's were identical to the last experiment, except that in all samples $[^{14}C]$ serine replaced $[^{3}H]$ and $[^{14}C]$ arginine.

The results are shown in Fig. 67. The patterns representing the two tubes containing infected cell sRNA and the two containing control cell sRNA have each been superimposed on the basis of the elution gradients of NaCl (Fig. 67(a) and (b) respectively).

The absorbancy patterns in all four samples are similar. The two radioactivity profiles corresponding to seryl tRNA's (Fig. 67b) from control cells are essentially replicates, and exhibit a minor peak of

incorporation at 0.45M and a major one at 0.56M NaCl.

Both preparations of infected cell seryl tRNA exhibit these two peaks of incorporation at 0.45 and 0.56M, but, in addition, a less well-defined species is evident at 0.60M. This strongly suggests the presence in infected cells of a tRNA which is able to accept serine, and which is not present in control cells. The pattern of incorporation found using the complete infected system was repeated in the system using infected cell tRNA and control enzyme. This supports the evidence presented in the last experiment on arginine "loading" that normal pre-existing cell ligase is able to function using this new tRNA fraction.

Attempts to form prolyl - tRNA in the above systems at this stage were unsuccessful.

(b) Formation of lysyl tRNA.

Lysine does not correspond to a triplet codon containing the CpG, GpC, CpC or GpG doublets. It was felt, therefore, since differences had been observed between populations of tRNA's after Herpes Simplex virus infection, that a test of the effect of infection on the synthesis of lysyl tRNA should be an excellent control in this system. Theoretical

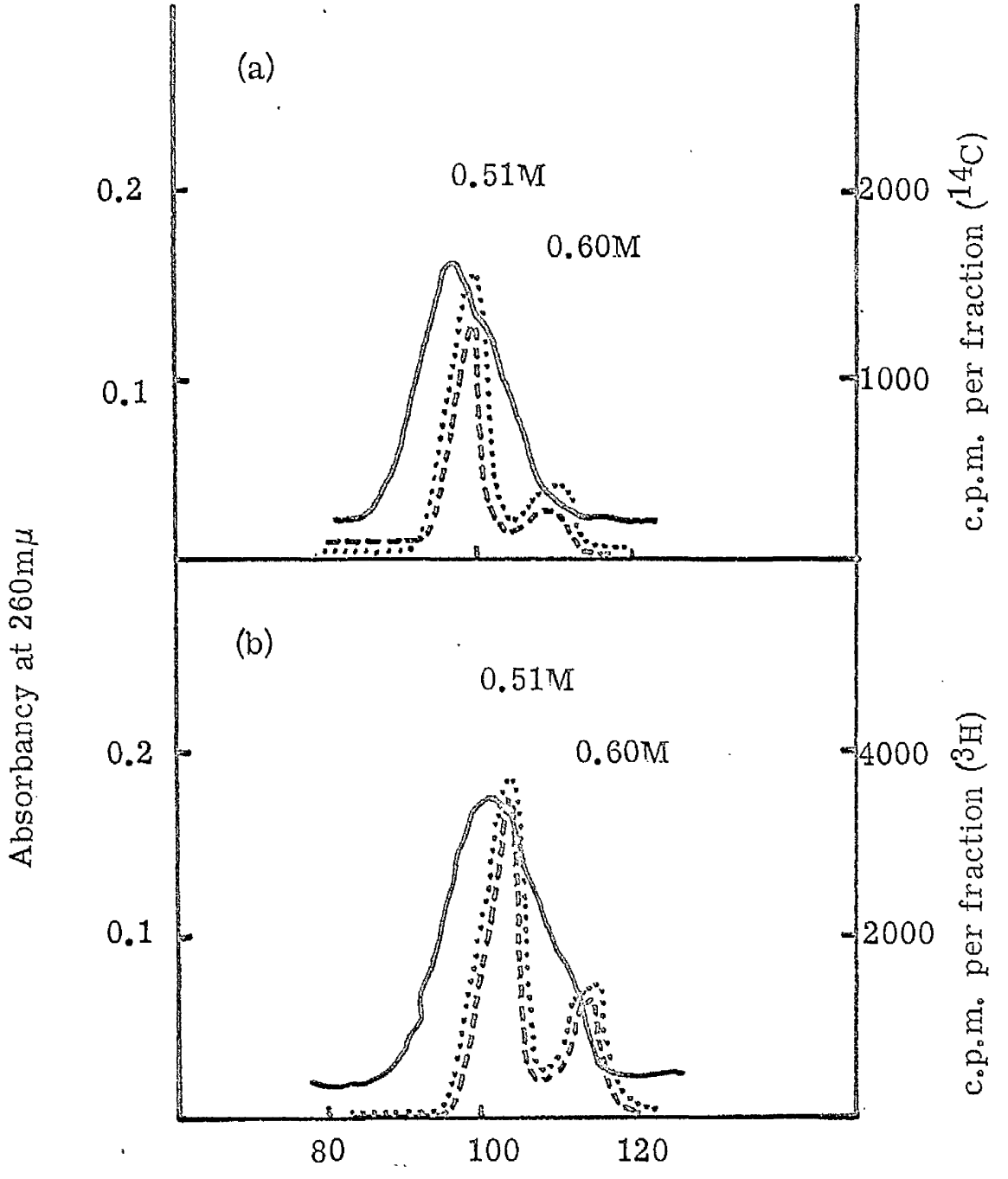
F I G U R E 63.

MAK column fractionation of Lysyl tRNAs. (a) Lysyl tRNA from Herpes Simplex virus-infected cell sRNA and BHK21 (C13) cell ligase (·····) plus lysyl tRNA from infected cell sRNA and ligase (----) (b) Lysyl tRNA from control cell sRNA and infected cell ligase (·····) plus seryl tRNA from control cell sRNA and ligase (----). The ligases were purified on DEAE-cellulose.

Elution with a linear gradient of NaCl gave rise to the peaks of radioactivity at the indicated NaCl concentrations.

——— Absorbancy at 260mμ

FIGURE 68


(a)
0.51M
0.60M
0.2
0.1
2000
1000
(b)
0.51M
0.60M
0.2
0.1
4000
2000
Absorbancy at 260mμ
c.p.m. per fraction (^{14}C)
c.p.m. per fraction (^{3}H)
80
100
120
Fraction Number

considerations predicted no alteration after infection. [^{14}C] lysine was incubated with control cell and herpes-infected cell enzyme and sRNA exactly as detailed in the last experiment. The method of isolation and fractionation of the product lysyl tRNA's on MAK was carried out exactly as before, and the absorbancy patterns and radioactivity profiles are in Fig. 66. Profiles from pairs of tubes were superimposed as described in the last experiment.

Control (BHK21 (C13)) cell lysyl tRNA, formed in the presence of enzyme from control and herpes-infected cells (Fig. 68a) elutes from MAK in two peaks; a major one at 0.51M NaCl and a minor one at 0.60M NaCl. This elution pattern is duplicated exactly by both preparations of infected cell lysyl tRNA (Fig. 68b) and this suggests that no alteration in the population of tRNA molecules able to accept lysine in BHK21 (C13) cells has taken place as a result of Herpes Simplex virus infection of these cells. This finding supports the specificity of changes observed in the arginyl and seryl tRNA populations (Results Sect. 20a, 21a).

22. FORMATION OF AMINOACYL tRNA USING UNFRACTIONATED ligase PREPARATIONS.

(a) DEAE-cellulose fractionation of the ligase

F I G U R E 69.

3 MAK column fractionation of Arginyl, Seryl and Lysyl tRNAs.

The aminoacyl tRNAs were formed using unfractionated ligase preparations.

Elution was achieved by a linear gradient of NaCl.

(a) Arginyl tRNA from control (.....) and infected (----) sRNA and ligase.

Control and infected patterns have peaks at 0.46M, 0.51M & 0.56M NaCl. respectively; a peak at 0.50M is confined to the infected preparation.

(b) Seryl tRNA from control (.....) and infected (----) sRNA and ligase. A peak of radioactivity at 0.60M NaCl is confined to the infected cell preparation.

(c) Lysyl tRNA from control (.....) and infected (---) sRNA and ligase.

The patterns are identical at 0.51M & 0.60M NaCl.

———— Absorbancy at 260mμ

FIGURE 69

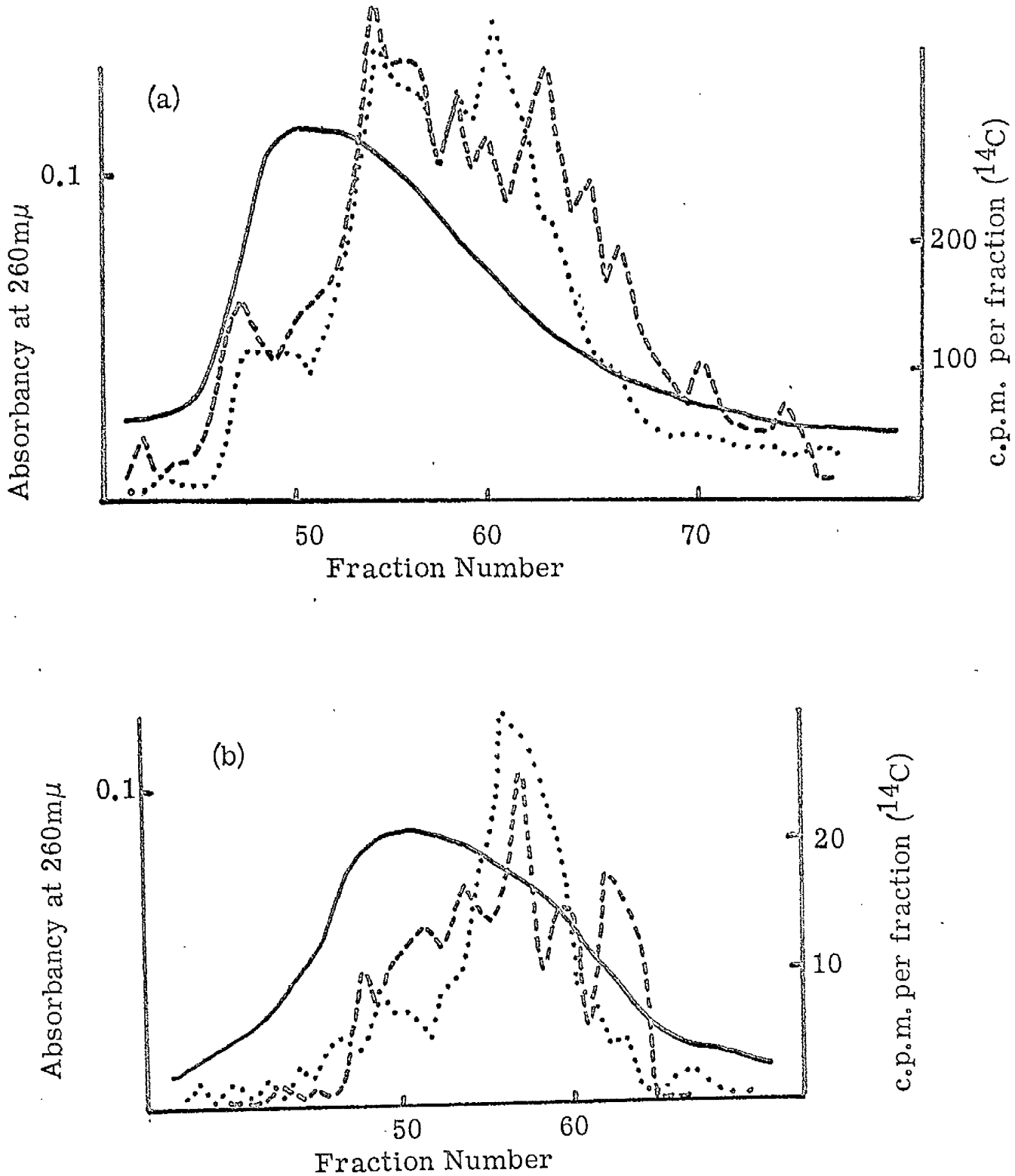
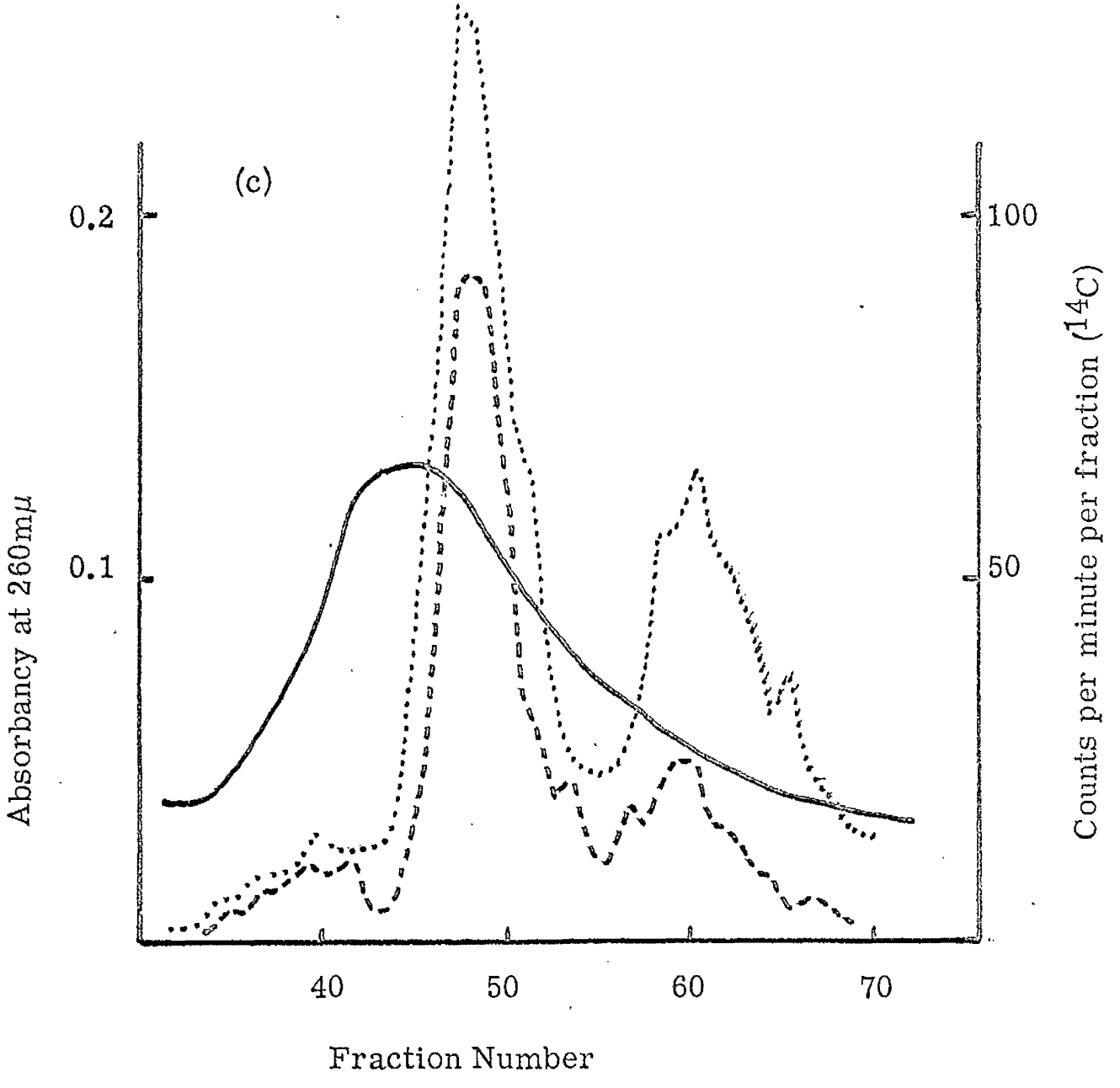

(a)
0.1
Absorbancy at 260mμ
200
100
c.p.m. per fraction (^{14}C)
50
60
70
Fraction Number
(b)
0.1
Absorbancy at 260mμ
20
10
c.p.m. per fraction (^{14}C)
50
60
Fraction Number

FIGURE 69


(c)
0.2
0.1
100
50
40
50
60
70
Absorbancy at 260mμ
Counts per minute per fraction (^{14}C)
Fraction Number

preparations has been used extensively in the last few experiments. This procedure, while removing sRNA from the enzyme preparation, has two unsatisfactory features (a) the time taken in fractionation may allow some of the enzyme activity to be lost and (b) some virus-specified enzyme (if such exists) may be fractionated out.

The following experiment was carried out using the crude ligase enzyme preparation as described in the methods section 10b. Six tubes were set up, all containing the standard incubation system for forming aminoacyl tRNA (Methods Sect. 11(b)). Three were given infected cell sRNA and enzyme, and three were given control cell sRNA and enzyme. One pair of each was completed with either [^{14}C] arginine, [^{14}C] serine or [^{14}C] lysine.

The conditions of incubation and isolation of the aminoacyl tRNA's have been described in full (Experiment 20a), and the MAK fractionation procedure was similar except for a less steep NaCl elution gradient: this, it was hoped, would produce better resolution of the aminoacyl tRNA's.

The results are shown in Fig. 69, and patterns representing samples incubated with one amino acid have

been superimposed. Fig. 69a shows that [^{14}C] arginyl tRNA formed using enzyme from BHK21 (C13) cells elutes from MAK at 0.46M (minor peak) and in broad major peaks at 0.51M and 0.56M respectively. Qualitatively these correspond well with earlier findings (Fig.65). [^{14}C] arginyl tRNA formed using infected cell material elutes also in these three positions, but, in addition, contains a fourth fraction corresponding to a broad peak at 0.58M NaCl. This last broad peak should be compared with a similar peak found in Fig. 64b.

The elution pattern of [^{14}C] seryl tRNA formed (Fig. 69b) using control enzyme and sRNA is identical with previously observed control profiles (Fig. 67b). Qualitatively the elution range of the infected seryl tRNA sample corresponds to the control material, with the exception of a peak at 0.60M NaCl. This corresponds to earlier findings (Fig. 67a). In this case the shallower gradient of elution has improved resolution.

The two lysyl tRNA samples are presented in Fig. 69c. Qualitatively, once more these demonstrate no apparent effect of infection on the population of lysine-specific tRNA's. It should be noted, however, that this analysis has revealed the presence, in both samples, of a minor component eluting at 0.45M NaCl in a broad peak.

F I G U R E 70.

MAK column fractionation of Prolyl tRNA.

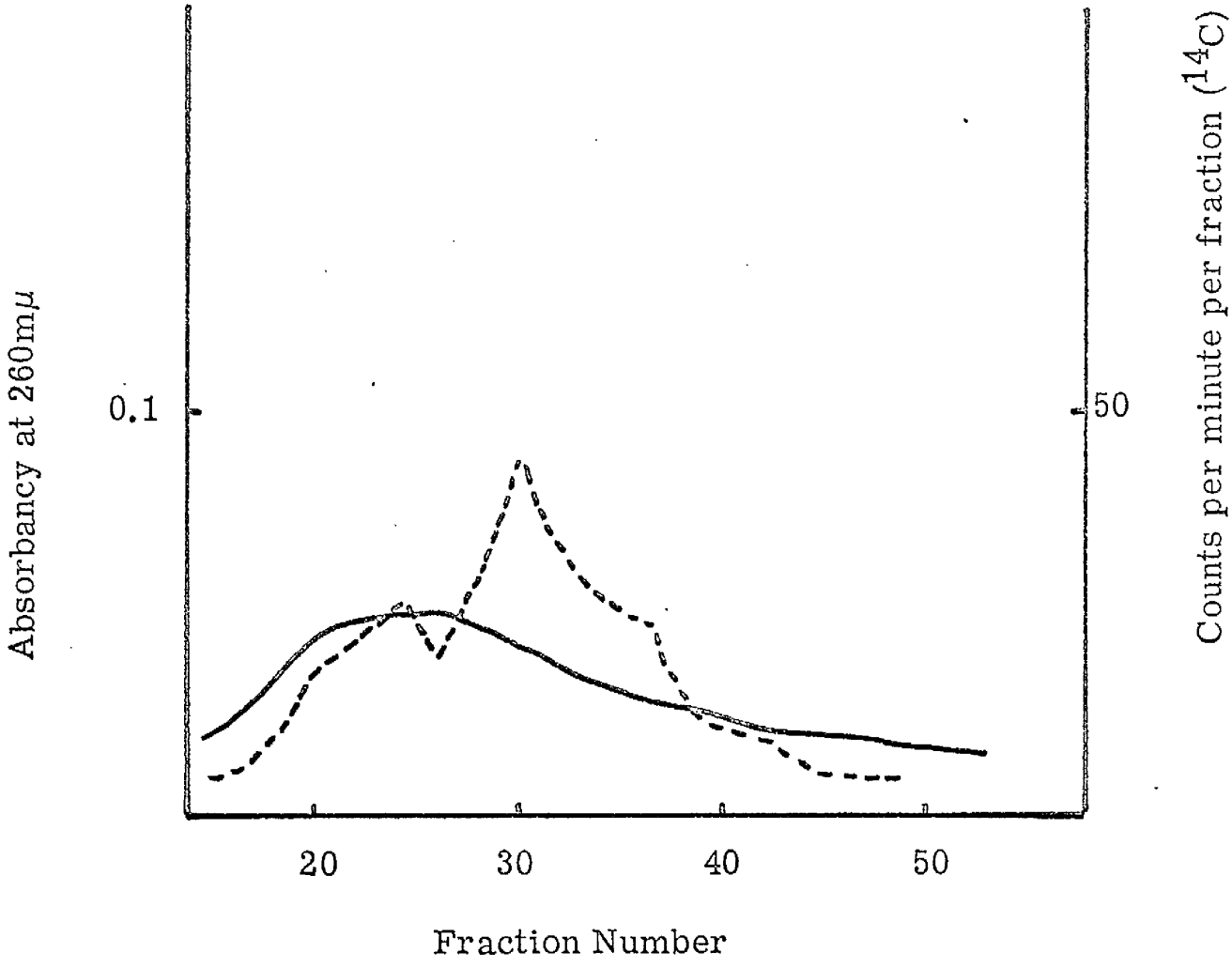
Unfractionated ligase was used, as was a linear eluting gradient of NaCl.

The two peaks of incorporation occur at 0.48M & 0.52M NaCl.

——— Absorbancy at 260mμ

– – – Radioactivity

FIGURE 70


Absorbancy at 260mμ
0.1
50
Counts per minute per fraction (^{14}C)
20
30
40
50
Fraction Number

(b) Formation of prolyl-tRNA.

This was carried out using BHK 21 (C13) sRNA and crude ligase preparation. Incubation with [^{14}C] proline, isolation of the product and fractionation on MAK were as described for the last experiment. A similar infected cell test was unsuccessful.

The result is shown in Fig. 70. The synthesised prolyl-tRNA elutes from MAK in two broad peaks, one at 0.48M (minor) the second at 0.52M.

23. DIGESTION OF AMINOACYL tRNA USING T1 RNase.

T1 RNase is a specific ribonuclease purified from Taka Diastase. It splits RNA phosphodiester bonds involving a guanosine residue, and this scission is quite specific: no rupture of an aminoacyl tRNA bond takes place (Ishida and Miura [358]). It is possible to fractionate the digestion products of T1 RNase action on columns of DEAE-cellulose, and (Ishida & Miura [358]) have obtained evidence of differences in structure between specific aminoacyl tRNA's using this technique.

It was decided to investigate this method with a view to using it to differentiate between control and infected cell aminoacyl tRNA preparations. [^{14}C] arginyl tRNA and [^{3}H]lysyl tRNA prepared as detailed in

F I G U R E 72.

Chromatography of T1 RNase digestion products of Aminoacyl tRNAs.

(a) Arginyl tRNA from BHK21 (C13) cells.

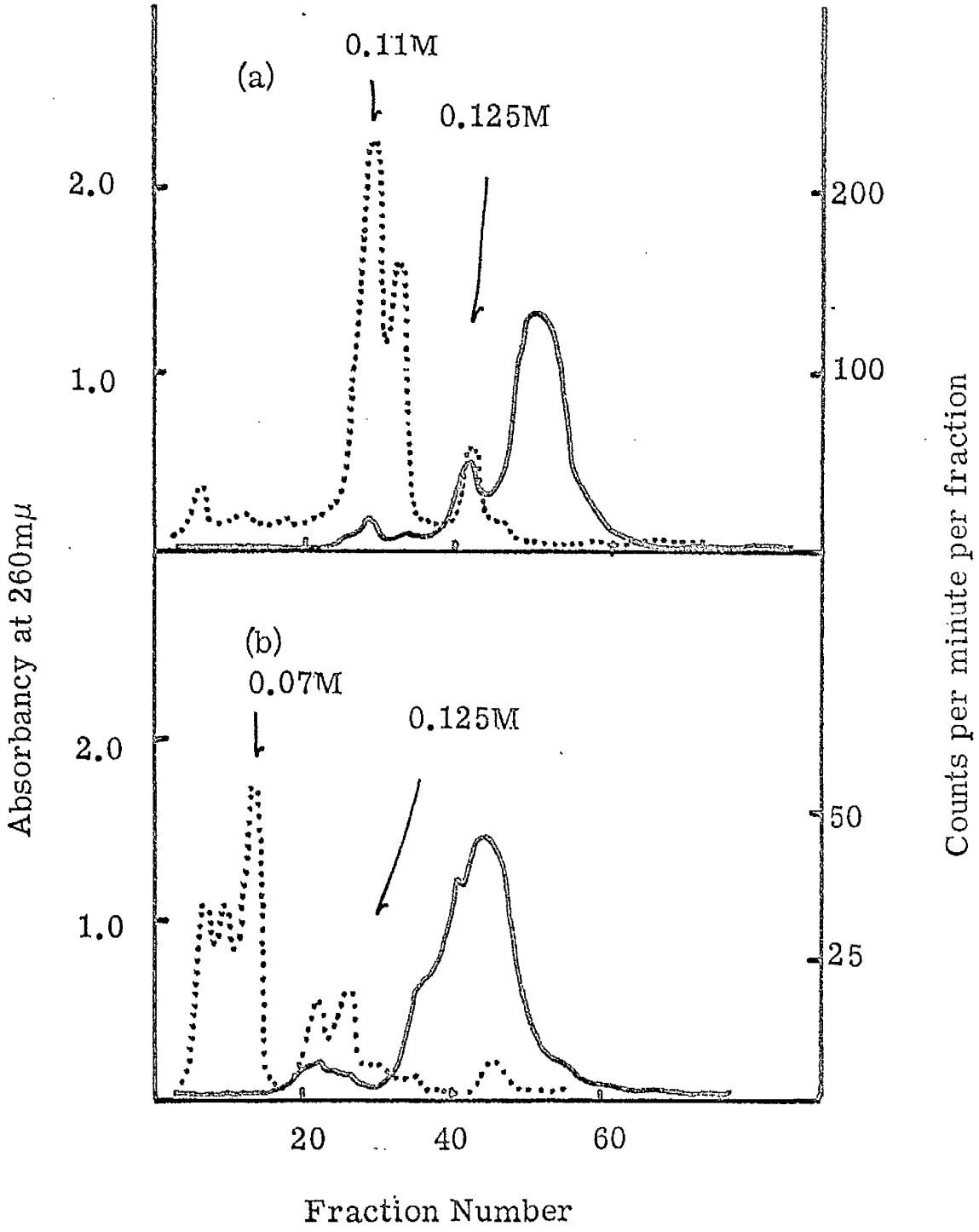
(b) Lysyl tRNA from the above cells.

A linear gradient of ammonium formate is used in elution.

——— Absorbancy at 260mμ

..... Radioactivity (^{14}C - Arginine)
(^{3}H - Lysine)

FIGURE 72


(a)
0.11M
0.125M
2.0
1.0
200
100
(b)
0.07M
0.125M
2.0
1.0
50
25
20
40
60
Absorbancy at 260mμ
Counts per minute per fraction
Fraction Number

the last experiment were incubated for four hours at 37° in the presence of purified T1 RNase (500 units/ 500ug. sRNA) at pH 5.5. Both digests were applied to a 10cm. x 1cm. column of DEAE-cellulose and eluted with a gradient of ammonium formate (Methods Sect. 11(c)). Each fraction (3ml.) was assayed for absorbancy at 260 mu and then evaporated to dryness at 150° in glass vials.

After addition of Toluene-based scintillator (Methods Sect. 6(h)(ii)) (10ml.), the fractions were assayed for radioactivity in the Nuclear Chicago Liquid Scintillation Spectrometer. Fig. 72a gives the digestion pattern of $[^{14}C]$ arginyl tRNA. All of the radioactivity represents fragments of RNA with amino acid attached - in other words, only the oligonucleotides corresponding to the amino acid - acceptor end of the tRNA can be picked out.

There appear to be three separable such terminal oligonucleotides in the BHK21 (C13) cell arginyl tRNA population, and these elute from DEAE-cellulose at 0.11M, 0.125M, and 0.170M NH_4OOCH respectively. There exist at least two such terminal oligonucleotides after T1 RNase digestion of $[^{3}H]$ lysyl tRNA (Fig. 72b). These elute at 0.07M and 0.125M NH_4OOCH respectively; there may be more than two species here, but the

radioactivity is too low to place these significantly.

It seems, then, that this method provides a means of differentiating between two aminoacyl tRNA species: it therefore ought to be useful in fractionating tRNA molecules derived from significantly different genomes (e.g. BHK21 (C13) and Herpes Simplex virus).

SECTION IV.

DISCUSSION

1. THE ANIMAL VIRUS GENOME.

There are two key facts about viruses from which all consideration of their structure and functional organisation must proceed. The first is that the essential infective agent of all viruses is a high molecular weight nucleic acid component. This may be RNA or DNA, but not, as far as is presently known, both. The second fact is that the infective nucleic acid is contained in a protein coat which serves to protect the nucleic acid until a susceptible host is encountered. This protein probably has a biochemical significance for the virus particle in addition to its structural role.

The viral genome has the ability to pre-empt the synthetic machinery of the host cell for the purpose of production of more virus. This may be accomplished, possibly not in identical ways, by DNA and by RNA.

The DNA or RNA virus genome may be one of a variety of size values and therefore of protein-specifying potential, provided that all of the nucleic acid is functional in this respect.

Viruses, then, may be conveniently classified according to the criteria given above, that is, in terms of the amount and type of information carried by the

nucleic acid.

1.
(a) RNA-containing Animal Viruses.

The RNA-containing animal viruses which have been studied in greatest detail are the smallest ones e.g. the Picornaviruses - EMC virus, poliovirus, etc. These have single-stranded RNA genomes of about 2×10^6 m.w. units, sufficient to code only for a few proteins, two of which have been characterised - the coat protein and the RNA-primed RNA polymerase which the virus uses to replicate its RNA.

The chemical basis of the cell-virus interaction is not well understood, but (Philipson & Lind [286]) have succeeded in obtaining a soluble lipoprotein receptor substance from erythrocyte membranes, which reacts *in vitro* with ECHO-7 virus: this culminates in the release of viral RNA. Not all virus adsorbed to the host cell is uncoated (e.g. poliovirus (Fenwick & Cooper [287])) and a specific mechanism involving host recognition may be active for a range of virus particles.

The infectivity of the isolated viral RNA has been studied to a large extent, and recent work indicates that it depends on the structural integrity of the EMC RNA molecule, and is abolished by a single 3', 5' -

phosphodiester scission (Montagnier & Sanders [288]).

The invading virus particle not only has the property of initiating its own synthesis, but also of inhibiting synthesis of host protein and nucleic acid, even under conditions preventing virus synthesis (Holland [289]) (poliovirus). (Holland and Peterson [290]) suggest that poliovirus infection halts host RNA synthesis while (Haselkorn and Preid [291]) visualise a competition for ribosomes between viral RNA and host messenger RNA. These two mechanisms may exist simultaneously.

There are several reports (e.g. (Tobey [292])) (Mengovirus) of the production of polysomes in small RNA virus-infected cells which contain viral RNA. This, and other evidence, suggests that the viral RNA is able to act directly as messenger RNA in its own protein synthesis.

That the single-stranded RNA of the infective particle is replicated via a double-stranded intermediate conforms to the known mechanisms of nucleic acid duplication. This double-stranded RNA has been isolated from EMC and poliovirus-infected cells (Montagnier & Sanders[293]; Baltimore et.al.[294]), and has been formed *in vitro* using the isolated RNA-primed

RNA polymerase (Baltimore [295]). The significance of such an "intermediate" molecule has been questioned, in an analogous bacterial system (Haruna & Spiegelman [166]).

Two group of viruses which are larger than the Picornaviruses by virtue of their more complex coat morphology alone are the Arboviruses and Myxoviruses e.g. WEE virus and influenza virus, respectively.

The Arboviruses infect cells and replicate in a fashion similar to the Picornaviruses: the situation with Myxoviruses however is not clear, since enzymes contained in the infective particle may play an important role in the infective process.

The RNA isolated from the Arboviruses is infective and this infectivity may depend upon the integrity of regions of intramolecular base pairing (Levintow [332]). This type of secondary structure may also exist in Myxovirus RNA (Sokol et.al. [333]) but this RNA has not been consistently found to be infective when isolated.

The genetic material of the Reoviruses is double-stranded RNA (Gomatos & Tamm [334]). This RNA is much larger (10^7 m.w. units) than the other classes or viral RNA, and interferes with cell DNA synthesis but not with total RNA or protein synthesis in the infected cell (Gomatos & Tamm [139]). It has not been

isolated in an intact form (Crawford [297]).

The isolated RNA is able to prime the action of DNA-dependant RNA polymerase *in vitro* to give a product that is partially RNase-resistant (Gomatos *et.al.* [150]): if this situation holds *in vivo*, then the synthesis of a specific RNA-replicating enzyme in Reovirus-infected cells may not be a necessary event.

These viral RNAs apparently contain no nucleosides other than A, U, C and G.

1.
(b) DNA-Containing Viruses.

The largest of the DNA-viruses, the Poxviruses, are also complex chemically. Their genome is double stranded DNA, and this has been isolated from vaccinia virus by (Joklik [298]) as a unit of 80 x 10^6 m.w. units; chemical estimation of the nucleic acid content of the vaccinia virus particle, however, gives a value of 160 x 10^6 m.w. units. However, no virus has yet been isolated whose genome has been certainly demonstrated to comprise more than one nucleic acid molecule, and (Joklik [298]) feels that the isolated DNA of vaccinia virus represents a half-molecule.

Infection of a cell with the vaccinia virus particle is mediated through an "uncoating enzyme" which

is present in infected cells. This enzyme specifically removes the coat from vaccinia virus and leaves the infective DNA core (Joklik [299]). This enzyme has been utilised by (Abel and Trautner [300]) to isolate DNA which will infect the bacterium B. subtilis and produce complete vaccinia virus particles.

This last fact supports the universality of the genetic code and the similarity of macromolecular biosynthesis in animal and bacterial cells. Although vaccinia virus has a large complement of DNA, about one tenth that of Escherichia coli, and therefore great protein-specifying capacity, very little work has been carried out in this direction.

The possibility remains that rabbitpox and vaccinia viruses contain 0.1 to 0.2% of RNA (Joklik [301]): this amount could code for approximately twelve proteins, if present, and this potential is twice that of a Picorna-virus, for example.

The members of the Poxvirus group have DNA base compositions which are alike and of the order of 40% G plus C (Joklik [298]).

The Adenoviruses constitute a group exhibiting marked host specificity and containing only DNA and protein.

The genomes of most groups of animal viruses have many similarities but the DNAs of Adenoviruses, apart from being double stranded, have few common features. For example, (Wasserman [311]) has noted that five human Adenoviruses behave differently to inactivation by ultraviolet irradiation or nitrous acid, and (Lacy and Green [304]) have shown that the DNA's of several types of human Adenoviruses hybridise with each other only to the extent of 20 - 40%. This degree of genetic diversity has been observed within much larger groups e.g. the bacteria and the vertebrates (Hoyer et.al. [305]).

The non-tumourigenic Adenoviruses have a larger complement of DNA per virion than have the tumourigenic Adenoviruses: this difference represents a coding potential of 3 proteins. Adenovirus DNA has a molecular weight of 15 x 10^6 m.w. units, and appears to have no unusual base constituent (Green [306]).

Thus far, only Adenoviruses type 12 and 18 have produced tumours experimentally (Green & Pina [309]) and these two types have base compositions of 48 - 49% G plus C. The remainder of the Adenoviruses have base compositions between 56 and 61% (Pina & Green [310]).

(Green [306]) raises the possibility of there existing a ring form of Adenovirus type 2 DNA, but the evidence

for this is not conclusive. The functional capacity of adenovirus-DNA has not been studied.

The Papovaviruses are a group of small DNA viruses, some of which are tumourigenic. These include polyoma and the papilloma viruses, the most intensively studied members of the group.

Polyoma DNA has a G plus C content of 49% (Crawford [33]) and can exist as a twisted, circular double-helix (Vinograd et.al. [45]). Preparations of Shope and human papilloma virus DNAs also contain circular double-stranded DNA molecules (Crawford [312]) and have DNA contents of 3 to 4 x 10^6 m.w. units.

The DNAs of these two Papovaviruses have been shown to be infective after isolation, as has the DNA of SV 40 virus (DiMayorca et.al; Ito & Evans; Gerber [313]). Both the ring and the linear forms of polyoma virus DNA have the ability to infect and transform cells (Crawford et.al. [314]). No virus-specified or induced enzyme protein has been positively identified in a Papovavirus-infected cell.

2. HERPES SIMPLEX VIRUS DNA.

Herpes Simplex virus is the most studied member of the virus group which contains pseudorabies, equine abortion and varicella viruses.

Russell and Crawford [315]) have shown that herpes DNA is double stranded, and has a base composition of 68% G plus C. This result disagrees with the estimate given by (Ben-Porat and Kaplan[316]) of 74% G plus C, and with the recent estimate of 65% ± 2.8% G plus C furnished by (Lando et.al.[317]). This present work, albeit utilising the methods employed by the first group above, estimates the G plus C content of Herpes Simplex virus to be 68 - 69%.

A value of 65% could be obtained if viral DNA was contaminated with host DNA in the experiment of (Lando et.al.[317]). This contamination would have to be of the order of 17%, however, if we assume the true value of the DNA composition to be 68% G plus C. The high value (74%) obtained by (Ben-Porat and Kaplan[316]) is most probably due to the false assumption that the specific activities of ^{32}P-labelled nucleotides from the DNA were equal under their experimental conditions.

Recent work (Subak-Sharpe et.al.[318]) on nearest neighbour frequency analysis of herpes DNA has given an estimate of 64 - 65% for the G plus C content of the pure viral DNA. This value is low, and yet it cannot be accounted for by host contamination, as this possibility has been eliminated.

The value of 77% G plus C for herpes DNA obtained in this work (Results Sect. 3d) using Tm measurements in low ionic strength is certainly too high. The value was calculated using data derived from bacterial DNAs, and may emphasise a physical difference between these two types of DNA.

The largest fragment of herpes DNA so far isolated is 50 - 60 x 10^6 m.w. units (Russell & Crawford [35]), and, since no accurate measure of the total DNA content of the virion is available, it cannot be conclusively stated that herpes virus contains a single molecule of DNA. The protein-coding potential of such a DNA molecule is, however, large, and sufficient for the specification of over one hundred proteins. This possibility, and the genetic behaviour of such a large DNA molecule of unusually high G plus C content, will be discussed later.

Herpes Simplex DNA is apparently not infective unless it is tested in the presence of a small amount of "helper" virus (Results Sect. 3a). However, since intact DNA molecules may not have been used in such assays, this situation may not be biologically significant. No evidence has been presented for the presence of unusual bases in or the possibility of a ring form of

Herpes Simplex virus DNA.

3. RNA SYNTHESIS IN CULTURED MAMMALIAN CELLS.

Because of the relative simplicity of the technique, many experiments on the synthesis of RNA in cell cultures have utilized the uptake of radioactive precursors into RNA as a measure of synthesis of the nucleic acid. Early work in this field (Thomson et.al. [319]), however, indicated that, in L cells, part of the newly synthesised RNA is subsequently broken down, and (Goldstein & Micon [320]) that, in human amnion cells, a one hour pulse of [^{3}H] cytidine results in nuclear labelling only. This label was subsequently shown to be transferred to the cytoplasm. Further work of this nature (Feinendegen et.al. [321]) indicated that the chromatin is most rapidly labelled in such an experiment, followed closely by the nucleolus. The work of (Shatkin [322]) with Hela cells reinforces the view that synthesis of RNA in cultured cells is largely nuclear and DNA-dependent.

(Scherrer and Darnell [181]) made the first careful analysis of the RNA components synthesised in a tissue culture cell (Hela) under conditions of a short pulse of radioactive precursor. Two fairly well resolved

components detectable by radioactivity alone sediment at 50S and 40S in sucrose gradient analysis. Three other RNA components at 30 - 32S, 18S and at about 4S were also typically present. The 32S and 18S components are constituents of ribosomal RNA while 4S material contains the tRNA of the cell (Harshaw et.al. [324]). The 50S component is the most rapidly labelled RNA of the cell, followed closely by the 40S fraction (Scherrer & Darnell [181]). This pattern of pulse-labelling of whole cell RNA has been observed in L-cells by (Rake and Graham [325]).

(Scherrer, Latham and Darnell [182]) have shown that, in Hela cells, most of the 50S and 40S RNA is high molecular weight ribosomal precursor material and (Girard et.al. [327]) that, in the presence of actinomycin D, some of this high molecular weight RNA is transferred to the cytoplasm as intact (74S) ribosomes, while the remainder is confined to the nucleus as ribosomal RNA. About one third of the total nuclear incorporation of radioactive uridine is, in this system, degraded to acid-soluble material after 30 minutes. This fraction is probably identical with the "metabolically unstable" RNA described by (Harris [328]) who proposes that it consists of RNA overproduced and not required by the

cell.

Messenger RNA has been identified in Hela cells with a polydisperse fraction of rapidly-labelled RNA which has a DNA-like composition and associated with ribosomes to form polysomes (Girard et.al. [329]). (Rake and Graham [325]) studying the kinetics of labelling of RNA in L cells, speculate that 50S and 40S RNA contain two sequential precursors of ribosomal RNA, and that the 50S RNA also gives rise to a fraction, at least, of 4S RNA.

More recently, (Penman [330]), using novel methods to fractionate cytoplasm and nuclei in Hela cells, has shown that 45S rapidly-labelled RNA breaks down into 16S ribosomal RNA, which enters the cytoplasm, and 38S ribosomal RNA which itself is altered to 28S ribosomal RNA without loss of RNA. No 16S ribosomal RNA can be isolated from Hela cell nuclei. These results are in broad agreement with the observations in L cells (Rake & Graham [325]).

Rapidly-labelled RNA of FL cells (Yoshikawa-Fukada et.al. [331]) has been fractionated on MAK columns, and two peaks of racioactivity which the authors suggest correspond to messenger RNA and ribosomal precursor RNA have been noted.

4. RNA SYNTHESIS IN BHK21 (C13) CELLS.

BHK21 (C13) cells were pulse-labelled with [^{3}H] uridine under conditions similar to those mentioned above. A pulse time of 30 minutes gives rise, in extracts of whole cells, to a pattern of incorporation into RNA similar in many respects to that observed in Hela cells and L cells (Scherrer & Darnell [181]) (Rake & Graham [325]). This pattern (Fig. 43a) shows peaks of incorporation at 45S and 35S, a small amount of labelling at 18S (possibly some ribosomal RNA synthesis) and 4S incorporation. In addition, there is evidence of some radioactive material sedimenting faster than 45S RNA which is probably pelleted on the bottom of the centrifuge tube.

It should be noted also that the base-line of the radioactive incorporation is not zero in Fig. 43a, but falls minimally to 25% of the peak level of labelling. This may, of course, be a result of merging of the RNA components due to diffusion, but is, more probably, indicative of the presence of polydisperse rapidly-labelled RNA components.

A longer time of pulse labelling with [^{3}H] uridine (1 hour) gives rise to a radioactive RNA pattern only minimally altered (Fig. 71a) from that obtained after

30 minutes labelling. In this case, however, a significant amount of ribosomal RNA labelling has taken place. Also, owing to the nature of the centrifugal analysis, resolution of 45S and 35S RNA has not been effected; nevertheless, it is readily apparent that there exists in these cells RNA larger than 45S, whose Svedberg coefficient may be as large as 55 - 60S. This probably corresponds to the sedimented material observed earlier (Fig. 43a).

After two hours exposure to [^{3}H] uridine (Fig. 71d), BHK21 (C13) cells contain this label in peaks corresponding to ribosomal RNA and 4S RNA. Very little incorporation into RNA heavier than 28S can be observed. This can be explained in terms of the added radioactive uridine being initially incorporated rapidly into RNA (35S and 45S largely) and not being subsequently degraded to a large extent to acid-soluble material capable of acting as precursor for additional RNA synthesis. Furthermore, this suggests, but does not prove, that if the above situation holds, that 45S and 35S RNA break down to give stable RNA (ribosomal).

In short, it seems reasonable to propose that the conditions and purpose of RNA synthesis in BHK21 (C13) cells are very similar to those existing in other cultured mammalian cell lines: the pattern of synthesis

has been shown to be identical.

This section of the experimental work was undertaken to establish with confidence a pattern of RNA synthesis typical to BHK21 (C13) cells. This was a necessary preliminary to (a) examining the effect of Herpes Simplex virus infection on cell RNA synthesis and (b) characterising virus-specified transcription.

5. RNA SYNTHESIS IN VIRUS-INFECTED ANIMAL CELLS.

(a) RNA viruses.

The infective RNA of single stranded RNA viruses is able, in infected cells, to act as messenger RNA in protein synthesis. This has been suggested by indirect evidence, e.g. in Mengovirus-infected L cells (Tobey [292]). The search for specific proteins produced by this viral messenger RNA has not been extensive, but, e.g., an RNA-primed RNA polymerase whose synthesis is attributed to the direction of the virus has been described in the above system (Baltimore and Franklin [336]) and probably exists in all such virus-infected cells.

The only double-stranded RNA animal viruses so far isolated, the Reoviruses, have not been characterised with respect either to their direction of specific protein synthesis or to the products of this synthesis, but

their replication is inhibited by actinomycin D (Gomatos et.al. [150]). These authors have reported that Reovirus RNA is able to prime DNA-dependant RNA polymerase: this has been refuted by (Shatkin [337]).

5.
(b) DNA viruses.

The first reports of synthesis of characterised RNA in DNA virus-infected animal cells were published contemporaneously by two groups of workers (Becker and Joklik [100]; Salzman et.al. [338]). These reports showed that vaccinia virus replicated in the cytoplasm of infected Hela cells and that there was a rise in cytoplasmic RNA synthesis after inspection.

This new RNA was determined by base composition and by hybridisation experiments to have been transcribed from the viral DNA. At the same time, host-specified RNA synthesis, largely nuclear, was observed to fall after a lag period of between 4 and 5 hours. The virus-coded RNA was shown to be heterogeneous with respect to size and, by the first group of workers, to increase in size as infection proceeded: its maximum size was between 10 and 30S. This RNA was shown by these workers to associate rapidly with ribosomes to form polysomes: they have assigned the properties

of a messenger RNA to this virus-produced material. More recent work has tended to confirm this view (Shatkin et.al. [369]), and (Jungwirth and Joklik [370]) suggest that the mRNAs coding for the "early enzymes" formed in vaccinia-infected cells are remarkably long-lived.

The vaccinia virus genome is relatively large, and therefore has the ability potentially to code for a large number of proteins. One of these, the viral coat protein, is formed around the viral DNA in a stepwise process and two-thirds of the produced viral DNA is not coated (Joklik and Becker [371]). A DNA nucleotidyltransferase and a thymidine kinase have been shown to be vaccinia-induced (Jungwirth and Joklik [370]; Kit and Dubbs [376]); and several deoxyribonuclease activities have been shown to rise after infection of cells with vaccinia and other DNA-containing animal viruses (McAuslan et.al. [372]). It is possible that at least part of these last activities is under the direct control of the invading virus genome. A large part, then, of the genome of those viruses remains unaccounted for in terms of protein-coding ability.

6. THE INFECTION OF BHK21 (C13) CELLS WITH HERPES SIMPLEX VIRUS.

The classical lesion of Herpes Simplex virus infection comprises (i) distortion and disappearance of the nucleolus (ii) distribution of the chromatin around the periphery of the nucleus and (iii) development of intranuclear inclusion bodies (Cowdray [373]). It is generally held that the virus multiplies in the nucleus of infected cells (Morgan et.al. [194]).

The synthesis of virus-specified protein takes place almost immediately after infection and continues until the point of maximal production of progeny virus particles is reached (Russell et.al. [148]).

(Aurelian and Roizman [374]) have shown that a strain of herpes virus is unable to produce progeny virus particles in dog kidney cells, but infects Hela cells normally, and have correlated this with interferon production (Aurelain and Roizman [375]).

The present work has shown that in BHK21 (C13) cells infected with Herpes Simplex virus strain a, the overall rate of synthesis of RNA falls immediately under the conditions of infection employed. It has been shown that synthesis of rapidly-labelled RNA transcribed from the viral genome is initiated and reaches a maximum between five and seven hours after infection: during the

next three hours this synthesis falls to a very low level which is, nevertheless, maintained until at least thirteen hours after infection. It is significant that mature virus particles are produced between four and seven hours after infection. It is reasonable to predict that the virus-coded RNA performs an mRNA function in protein synthesis and its pattern of synthesis is compatible with the appearance in infected cells of herpes-coded proteins e.g. Thymidine kinase (Kit and Dubbs [376]). DNA nucleotidyltransferase and a deoxyribonuclease may also be synthesised under the direction of herpes simplex virus in infected hamster kidney cells (Keir et.al. [126]; Morrison and Keir [377]).

Herpes-coded rapidly-labelled RNA is polydisperse: the size range is from 10s to 30s, but this range apparently does not alter during infection, although it must be borne in mind that, especially during the initial stages of infection, the separation of host-specified RNA from virus-specified RNA is not definitive. The size pattern of RNA molecules transcribed from the viral genome is probably reflected in the synthesis of several different protein species. The 10s to 30s rapidly-labelled RNA is not present in substantial amounts in the cytoplasm of infected cells during 6 to 7 hours after

infection and the constant low level of synthesis of this RNA in the later stages of infection (10 to 13 hours) may be a consequence of asynchronous infection of the kidney cells. Reports of this work have been published (Hay et.al. [378, 379]).

The virus particle penetrates the host cell and is enclosed in a vacuole, from which it escapes after its outer coat has been digested (Epstein et.al. [380]). The time of penetration of the virus into the cell has been variously estimated to be 2 hours (Farnham and Newton [381]) and 7 minutes (Kiang and Wagner [382]) at 37°.

7. ALTERATIONS IN HOST-SPECIFIED RNA SYNTHESIS AFTER HERPES SIMPLEX VIRUS INFECTION.

It has been shown that the characteristic pattern of RNA synthesis obtained in BHK21 (C13) cells during a 30 minute exposure to radioactive precursor is profoundly altered as a result of infection by Herpes Simplex virus. Furthermore, this host-specified RNA synthesis is not observed under the above conditions in cells infected for 7 hours. Ribosomal RNA synthesis and sRNA synthesis also fall markedly during the above period, and, under these circumstances, it seems reasonable to suggest that many, if not all, the functions

of the host cell DNA are eliminated as a result of Herpes Simplex virus infection. It has been suggested tentatively that the virus is responsible for breakdown of the host cell DNA (Wildy et.al. [383]), but this breakdown may merely be a consequence of intracellular disorganisation.

The hybridisation experiments do not rule out the possibility that cell-coded RNA synthesis occurs after infection; however, it is equally possible that the virus and host cell DNAs contain relatively long base sequences which are identical.

8. SYNTHESIS OF TRANSFER RNA IN BHK21 (C13) CELLS BEFORE AND AFTER INFECTION WITH HERPES SIMPLEX VIRUS.

(a) The uninfected cell.

(Giacomoni and Spiegelman [353]) and (Goodman and Rich [173]) have suggested, from the results of DNA:RNA hybridisation experiments, that sRNA is transcribed from bacterial cell RNA: (Macfarlane and Fraser [173]) propose that a similar situation exists in Ehrlich ascites-tumour cells.

Evidence has been presented in this present study that BHK21 (C13) cell DNA codes for sRNA. This sRNA was purified exhaustively to remove all non-tRNA material from the preparation: however it is not possible

to state with complete confidence that the methods used accomplished this aim. It is therefore possible that the sRNA which was observed to form an RNase-resistant hybrid with BHK21 (C13) cell DNA was not tRNA, although we have repeatedly demonstrated the amino acid-acceptor ability of sRNA preparations. The criticism applies equally to the observations reported by the above workers, and there are technical difficulties which preclude the successful completion of an entirely definitive experiment.

However, there is indirect evidence (e.g. Ritossa and Spiegelman [175]) to support the DNA-origin of tRNA and this situation is generally held to apply to all living systems.

In the absence of firm knowledge of the heterogenicity of the BHK21 (C13) cell DNA population (or of the number of tRNA coding sites per molecule) it is difficult to make an estimate of the percentage of host DNA molecules involved in this transcription. Suffice it to say that this amount must be very small, probably not more than 0.02% of a cell DNA molecular unit. This figure has been derived from the results of DNA:RNA hybridisation experiments (Fig. 56 & 59).

MAK column fractionation of specific aminoacyl

tRNA from BHK21 (C13) cells has given evidence for the existence in this mammalian system of three arginyl tRNAs, three lysyl tRNAs, two seryl tRNA and two prolyl tRNAs. Two points must be considered in connection with these findings: (i) that two or more tRNAs may well carry the same anticodon and (ii) that two tRNAs corresponding to separate genetic codons are not ipso facto separated in the analysis used.

It is of interest that this analysis of the diversity of tRNA molecules in mammalian cells yields results similar to those obtained with other organisms e.g. yeast and several bacteria (Sueoka and Yamane [346]).

Fractionation of T1 RNase digestion products of specific aminoacyl tRNAs has also emphasised the heterogeneity of tRNA molecules from BHK21 (C13) cells, especially in respect of the nucleotide sequence near the amino acid-accepting terminal of the molecules. Three kinds of terminal sequence were observed in arginyl tRNA, and at least two in lysyl tRNA. It would be tempting to equate these results with the fractionation obtained on MAK columns, but such a comparison would not be wholly justifiable: however, the previously observed heterogeneity is emphasised.

Both sets of analyses reveal marked quantitative

differences between the members of a set of aminoacyl tRNAs. This may arise merely from the differential stability of each aminoacyl tRNA or may be attributable to the action of specific ligases, but, more probably, it reflects the composition of the cell tRNA population. If this last possibility obtains then it might be predicted further that the composition of the tRNA population of the cell is adjusted particularly to meet the cell's requirements.

8.
(b) Virus-specific synthesis.

DNA:RNA hybridisation experiments show that in Herpes Simplex virus-infected BHK21 (C13) cells there exists sRNA specified by the virus genome. This sRNA hybridises with the host DNA also, but to a markedly smaller extent. The criticism applied to the similar results obtained in uninfected BHK21 (C13) cells, and, indeed, relevant to all such experiments with sRNA, holds in infected cells. It is possible that the infected cell sRNA contains nucleic acid which is not tRNA.

It has been shown, however, that specific tRNA populations in BHK21 (C13) cells have been altered by the addition of at least one new species of tRNA as a result of infection of BHK21 (C13) cells. Furthermore,

these results are in agreement with the hypothesis presented earlier, based on purely theoretical considerations. While it is not justifiable to conclude unequivocally that Herpes Simplex virus is responsible for the transcription from its own genome of particular tRNA molecules, the results are most suggestive of it. It is worthwhile noting that this Herpes-coded tRNA is not, it is suggested, produced in order to allow virus-coded protein synthesis an advantage over host-specified activity, but occurs simply as a necessary response to the imbalance of the host tRNA population. (Sueoka and Kano-Sueoka [385]) have reported a specific structural modification of one, or possibly two, leucyl tRNA species in Escherichia coli B infected with T2 bacteriophage which may affect the transition from "early" to "late" protein synthesis in these cells, or may affect the translation of preformed most mRNA. Such a situation may also occur in the Herpes Simplex - BHK21 (C13) cell system, but it is clear from the results presented here that a much more extensive contribution to the sRNA of the infected cell is made by Herpes Simplex virus than could be accounted for by the above mechanism alone.

It may be calculated from the hybridisation

experiments, assuming asymmetric transcription and a viral DNA molecule of 60 x 10^6 m.w. units that 1.2% of the Herpes Simplex genome can form a specific complex with infected cell sRNA. If this represents tRNA, then between 10 and 20 tRNA species may potentially be formed from viral DNA. This estimate is, of course, approximate, but it is worthy of note that it is of the right order with respect to the considerations mentioned above in the Results Sect. 15.

As previously mentioned, some of the infected cell sRNA formed a specific hybrid with the host cell DNA. This may indicate (i) that there is homology between BHK21 (C13) DNA and Herpes virus DNA (this is not substantiated by hybridisation experiments using host cell sRNA and virus DNA) or (ii) that host cell-specified tRNA synthesis takes place at an accelerated rate after infection. It may also demonstrate the presence in infected cells of an RNA species whose size is similar to a tRNA molecule, but whose function is to control transcription of the host DNA; this species would be produced by the viral DNA and would be capable of at least some degree of binding to the host DNA.

The very recent observations of several groups (e.g. (Khorana [386]) (Matthaei [386])) have led to the assignment of almost all of the 64 possible triplet

codons in terms of protein synthesis (see Table 14). From this, and from the assignment of the probable anticodons in alanyl tRNA (Holley et.al. [76]) seryl tRNA's (Zachau et.al. [387]) and valyl tRNA (Baev et.al.[388]) (these are IGC, IGA and IAC respectively) has come support for the "wobble hypothesis" proposed by (Crick[389]). This states simply that there does not exist a tRNA molecule specific for each of the 64 triplet codons and that one tRNA molecule may recognise a set of up to four triplet codons equally well. This degeneracy involves only the terminal (5') nucleotide of a given triplet codon sequence. The hypothesis does not state that only one tRNA molecule is specific for one set of codons and, in fact, there is evidence to the contrary (e.g. (Zachau [387])).

Certain of the results in this thesis are not affected by and indeed have no bearing upon the "wobble hypothesis" (e.g. experiments with arginine and lysine) but if, for example, the finding of a virus-coded seryl tRNA is correct and if this tRNA is synthesised for the reason that has been predicted from theory, then this rules out types of wobble (A↔G or A↔G↔U↔C) existing in the serine system. The results obtained using proline, alanine and threonine also bear directly upon

the hypothesis, and so it is imperative that these results be confirmed and obtained unequivocally.

In short, the results given here are compatible, given certain limitations, with the "wobble hypothesis"; further results in this system may help to clarify the position of this hypothesis in the extremely important field of protein synthesis. Preliminary reports of this work have appeared. (Hay and Subak-Sharpe; Subak-Sharpe and Hay; Subak-Sharpe and Hay [390]).

9. BIOCHEMICAL EVENTS IN THE HERPES SIMPLEX VIRUS-INFECTED CELL.

The Herpes Simplex virion infecting its host cell rapidly causes a fall in the overall rate of RNA synthesis which has been shown to reflect the cessation of synthesis of host-coded rapidly-labelled RNA (probably ribosomal precursor RNA and mRNA) and of ribosomal RNA. The virus does not cause the breakdown of ribosomal RNA formed prior to infection. The synthesis of sRNA falls in infected cells after a time lag of 4 hours. The first appearance of virus-coded rapidly-labelled RNA (again probably mRNA) is at about 90 minutes after infection, this event following on the uncoating of the virus and the availability of its DNA for transcription: several virus-coded events involving protein synthesis

ensue e.g. elaboration of complement-fixing antigen (Russell et.al. [148]).

The virus DNA begins to be formed between 4 and 5 hours after infection, and infective virus one hour later. It is not clear from the present study at which stage during infection the virus-coded tRNAs are formed, but it seems reasonable to suggest that their synthesis should be an early rather than a later function after infection. Such production may explain the lag in the fall-off of overall sRNA synthesis observed.

Herpes Simplex virus appears to overcome the host in terms of protein-synthesising ability and it seems to use at least part of the host's machinery in directing its own protein synthesis.

HORIZONS.

This work has defined certain parameters in relation to synthesis of protein and of mature Herpes Simplex virus in the infected cell. It remains to be seen whether the rapidly-labelled RNA discovered after infection has a true messenger function, and for this reason, it must be tested for its ability to direct the incorporation of amino acids into specific polypeptide molecules: this is the criterion. It would also be of

interest to examine the ability of the virus' information to express itself in an environment devoid of the specific aminoacyl tRNAs which, it is speculated, it cannot do without. Such a situation may occur with respect to an RNA-containing animal virus, Rauscher virus, an oncogenic virus of mice (Zamecnik [392]).

Thus far, no attempt has been made to elucidate the mechanisms whereby the virus is enabled to overcome its host. It is tempting to speculate that competition for protein synthesising machinery between host and virus is resolved in favour of the virus, purely on the basis of a vast over-production of virus-specific informational molecules and the resulting mass action effect, but this hypothesis is not favoured by the results obtained: viral DNA is produced in quantity, but too late, while RNA is present in relatively small amount. This problem is extremely interesting and its solution is vital, not only to an understanding of the control of virus infection, but also to the solution of the complex paradoxes surrounding control mechanisms in mammalian systems themselves.

That Herpes Simplex virus DNA is a large molecule in terms of information storage leads to the question whether or not the whole virus DNA molecule is

functional in specific macromolecular synthesis. Current work on the proteins which occur only in infected cells does not resolve this problem, and, unless several cistrons appear more than once in the genome, much of Herpes Simplex virus DNA remains to be accounted for, either in terms of redundancy, or in terms of direction of specific synthesis. If, then, only certain stretches of the virus DNA contain information which is expressed, by what type of control is the remainder of the molecule suppressed ? This may be a function of the coat protein, but, at this time, this seems unlikely.

By means of kinetic studies on DNA:RNA hybridisation and by analysis of polysome patterns in infected cells, it is hoped that the problem of possible viral DNA redundancy may be resolved and that the question of temporal RNA synthesis after infection will be answered.

At present, we do not know which specific event initiates the destruction of a complex biochemical unit by a fragment of nucleic acid coated with protein. This is, perhaps, the most pressing problem of all.

SUMMARY.

1. A method has been developed for the extraction of DNA from Herpes Simplex virus, and from its host cell, BHK21 (C13). The method may successfully be applied to a variety of DNA-viruses and mammalian cells.

2. The isolated viral DNA has been demonstrated to be biologically active in certain transformation experiments, to be relatively homogeneous with respect to size and to have the characteristic melting profile of a double-stranded DNA molecule. Suitable conditions for the irreversible separation of the component strands of viral DNA or of host DNA have been established.

3. From melting temperature measurements and from estimations of the buoyant densities in CsCl of viral and host DNAs, the following values for the base compositions have been obtained: 68 - 69% G plus C for Herpes Simplex virus DNA and 41% G plus C for BHK21 (C13) DNA. The average size of a representative preparation of viral DNA was shown to be 2/3S.

4. Attempts to separate Herpes Simplex virus DNA and BHK21 (C13) DNA from a mixture of both by chromatography on methylated albumin columns were unsuccessful; this was probably due to size heterogeneity of both DNAs under the conditions employed.

5. Several methods of fractionation of RNA were investigated. Sucrose gradient analysis was chosen for its convenience and good resolving properties.

6. Factors affecting the isolation, in an undegraded state, of RNA from Herpes Simplex virus-infected and uninfected BHK21 (C13) cells were assessed. (i) the isolation procedure of Eason, Climo and Smellie was adopted (ii) a method of harvesting and initially lysing the cell suspension was developed. (iii) the use of Bentonite and redistilled phenol were found to be essential for (iv) dialysis was adopted as the preferred method of removal of small molecular weight material from the final product.

7. Rapidly-labelled RNA formed in BHK21 (C13) cells in a 30 minute-pulse experiment was consistently shown to comprise a 45S and a 32S component, with smaller amounts of synthesis of 18S RNA and 4S RNA.

8. The 45S RNA was shown to be derived from the BHK21 (C13) cell genome, and to be similar in base composition to 28S ribosomal RNA from these cells. Longer times of exposure of cells to radioactive precursor gave rise to a relatively larger incorporation of label into ribosomal RNA. This evidence and analogous work in other laboratories suggested that 45S (and

possibly also 35S) RNA contain ribosomal precursor material.

9. Infection of BHK21 (C13) cells with Herpes Simplex virus gave rise to an almost immediate fall in overall RNA synthesis which was correlated with the appearance of progeny virus in the same system.

10. Fractionation of rapidly-labelled RNA at several periods after infection demonstrated that synthesis of 45S and 35S RNA rapidly diminished but remained host-specific, and that an 18 - 23S RNA species was formed, maximally, at 5 - 6 hours after infection. 4S RNA continued to be synthesised after infection.

11. The 18 - 23S RNA component was shown, in hybridisation experiments, to have been transcribed from the viral genome. A small fraction of this RNA hybridised specifically with BHK21 (C13) DNA also.

12. Ribosomal RNA formed in uninfected cells was shown to be stable up to at least 6 hours after infection, while synthesis of ribosomal RNA in infected cells fell dramatically during this period.

13. The S values of ribosomal RNAs isolated from BHK21 (C13) cells were calculated to be 27S and 18S

respectively. These values did not alter after Herpes Simplex virus infection but were dependent upon the concentration of Mg^{2+} in the solvent.

14. Addition of Actinomycin C to the growth medium suppressed the synthesis of RNA in HEK21 (C13) cells before and after Herpes Simplex virus infection.

15. Theoretical considerations led to the conclusion that Herpes Simplex virus coded for synthesis of a population of specific vRNA molecules in an infected cell.

16. Exploratory experiments, with partially-purified and purified vRNA preparations, involving DNA:RNA hybridisation studies demonstrated that vRNA from HEK21 (C13) cells was able to form a specific hybrid only with HEK21 (C13) DNA, and that vRNA from infected cells hybridised with viral DNA and, to a certain extent, with host DNA.

17. An estimated 1.2% of the viral DNA is involved in vRNA coding, but attempts to quantitate the kinetics of production of the virus-specific vRNA were not fruitful. It was shown, however, that overall synthesis of vRNA did not diminish until 4 hours after infection.

18. Physical measurements on vRNA preparation revealed

no clear-cut difference between the uninfected cell and infected cell preparations.

19. Formation *in vitro* of aminoacyl tRNAs using extracts of uninfected and infected cells demonstrated the presence of three arginyl tRNAs, three lysyl tRNAs, two seryl tRNAs and two prolyl tRNAs in BHK21 (C13) cells. After infection with Herpes Simplex virus, a new arginyl tRNA and, possibly, a new seryl tRNA were elaborated.

20. Fractionation of T1 RNase-digestion products of aminoacyl tRNAs confirmed and extended the significance of the above patterns of aminoacyl tRNA synthesis in BHK21 (C13) cells.

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