DETECTION AND LOCALISATION OF VIRUS DNA AND RNA IN EUKARYOTE CELLS

by

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I declare that the work described in this Thesis is my own.

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SUMMARY

A characteristic feature of mammalian DNA virus transformed and tumour cells is the persistence of virus DNA, and usually but restrictively, its expression. This "genetic transformation" of individual normal cells may therefore form the basis of "phenotypic transformation" i.e. transformation as observed at the gross or morphological level.

The amount of virus DNA, which appears to be consistently associated with host cell nuclear DNA and therefore with chromosomes, is likely to be an important feature in itself and is an indication of the level at which virus-host cell interactions operate during either transformation or oncogenesis. The present work describes experiments which attempt to estimate the amount of virus-specific DNA sequences in Adenovirus transformed or tumour cells: chiefly by cRNA-DNA hybridisation techniques. By characterising the virus cRNAs, both at the transcriptional and hybridisation levels, and therefore increasing the resolving power of the cRNA-DNA hybridisation technique, at most 3-4 copies of virus DNA complementary to the virus cRNAs were found to exist per diploid quantity of host DNA. Furthermore, using in situ hybridisation - the resolving power of which has also been increased during the present work-it has been shown that these low amounts of virus DNA sequences most likely represent the values per individual cell. These results are consistent with a very basic level of virus restriction existing in the transformed or tumour cells studied.

The low amounts of virus DNA per individual transformed or tumour cell mean, in effect, that these sequences are difficult to detect by

the current techniques available: especially at the single cell level. Many of these virus DNA sequences, however, are transcribed *in vivo* and as such are amplified. Using a modification of the *in situ* hybridisation technique virus-specific RNA sequences have been detected in individual Adenovirus transformed or infected cells. Such a method opens up the possibility of detecting, for example, heterogeneity of virus-specific RNA transcription in tumours. Preliminary experiments, with this approach in mind, have demonstrated such a heterogeneity of messenger RNA transcription between cells in the same tumour.

CONTENTS

CHAPTER I

INTRODUCTION

Several mammalian DNA viruses are capable of transforming cells in culture. Such transformed cells usually display *in vitro* characteristics which are different from normal or untransformed cells: for example, the ability to grow to high densities when compared with appropriate control cells (Gallimore, 1974); the ability to produce colonies in semi-solid agar medium (Macpherson and Montagnier, 1964); and the predisposition to be agglutinable by various plant lectins at concentrations that fail to agglutinate normal cells (Inbar and Sachs, 1969).

Whether such transformation can be correlated with the persistence and expression of virus genes within the affected cell has, for a long time, been an interesting question in virus-cell interactions.

The virus genome is at least partially present in some virus-transformed cells as demonstrated by the presence of new transformation-specific proteins, or new virus-specific antigens. SV40 transformed cells, for example, express several virus implicated functions such as the SV40 T antigen (Black, *et al.*, 1963; Green, 1970) and the tumour-specific transplantation antigen(s) (TSTA) (Habel, 1965; Green, 1970). Similarly, Adenovirus transformed cells synthesise at least two types of virus-implicated antigens: T-antigen(s) and TSTA antigen(s). And as far as have been tested, all lymphoblastoid cell lines of human origin contain complement-fixing antigens specific for Epstein-Barr virus (EBV) (Pope *et al.*,1969; Vonka *et al.*, 1970).

That the new antigens in virus transformed cells are the products of virus or virus-mediated gene expression is supported by a good deal of circumstantial evidence; for example, the persistence of virus DNA (e.g. Klein, 1975; Botchan et al., 1974; Westphal and Delbecco, 1968; Green et al., 1970) and its transcription (e.g. Ozanne et al., 1973; Botchan et al., 1974; Green, 1970) appear to be general phenomena.

Many of the mammalian DNA viruses which can cause transformation also possess oncogenic potential: i.e. the ability to cause tumours in appropriate hosts. But the realisation of this potential can be influenced by a variety of factors including the nature of both the virus and the host. Immunosurveillance and tolerance by the host is a case in point (see Klein, 1975b for example). Reflecting this are the human Adenoviruses which have been classified as highly, moderately, or non-oncogenic in vivo in hamsters (Huebner, 1967); and while some serotypes appear to be non-oncogenic on this basis they may nevertheless transform cells in vitro (Freeman et al., 1967; McAllister et al., 1969) which may then produce tumours following inoculation into immunosuppressed hosts (Gallimore, 1972).

As in DNA virus transformation new internal and surface antigens are detectable in the tumours and in the cell lines derived from them. These tumour cells retain their malignant character, the capacity to synthesise the new virus-specific antigens, and the appearance and in vitro behaviour of virus transformed cells even through many cell generations on cloning.

Some tumours which have arisen in vivo without experimental manipulation or deliberate induction can also be associated with

oncogenic DNA viruses, there being high antibody titres to the virus in question in individuals suffering from the tumour. For instance there is an association between antibodies to Herpesvirus type 2 and cervical neoplasia (Nahmias et al., 1970; Royston and Aurelian, 1970). Also African Burkitt's lymphoma and certain nasopharyngeal carcinomas are consistently associated with high EBV antibody titres (Klein, 1975).

The appearance and persistence of new virus-specific antigens is consistent with persistence and the expression of the virus genome as in DNA virus transformation. This is further substantiated by the detection of virus-specific RNA and DNA within a variety of virus-implicated tumours; for example Adenovirus-induced tumours (Fujinaga and Green, 1966; 1967; 1968; Green, 1970) SV40-induced hamster tumours (Oda et al., 1972), Polyoma-induced tumours (Axelrod et al., 1964) and EBV-implicated tumours (Zur Hausen et al., 1972; Nonoyama et al., 1973; Pagano, 1974; Klein, 1975).

Thus the general similarities between transformed cells and tumours, for example their growth characteristics in vitro may reflect a basic underlying similarity in the presence of virus DNA and its possible expression. Experiments described in this thesis were designed to elucidate the state of the virus DNA and its transcription into RNA in individual virus-transformed or tumour cells.

By way of introduction to these particular experiments there follows a general account of virus DNA in virus-transformed or tumour cells. Virus-specific RNA in virus-transformed and tumour cells is dealt with in Chapter IV.

STATE OF VIRUS DNA IN MAMMALIAN DNA VIRUS-TRANSFORMED AND TUMOUR CELLS

One of the striking features of mammalian DNA transformation and tumourogenesis is, in general, the lack of production of virus particles. This is in marked contrast to permissive systems (where productive cycles are initiated and completed) which suggests that virus maturation is blocked at some stage in the transformed or tumour cell. There are several stages of virus maturation where blockage or interference could occur, for example at the level of virus assembly, translation, transcription, or DNA replication. In most transformed or tumour cells not all the virus-specific proteins or RNA sequences that appear during a normal productive cycle are present. The virus-specific RNA, for example, is usually confined to those sequences transcribed previous to virus DNA replication in the normal productive cycle (Green, 1970). Selectivity in translation and transcription, however, could also be explained by deletions of the appropriate regions of the virus genome. For the vast majority of transformed cells this appears unlikely in view of the fact that in these cells some infectious virus can be rescued by cell fusion (Watkins and Dulbecco, 1967; Gerber, 1966; Kowprowski et al., 1967) and/or by induction by chemical means (e.g. Glaser and Rapp, 1972). Nevertheless some transformed cells could possess incomplete genomes on this basis since attempts to rescue infectious virus by these means have usually been unsuccessful. Adenovirus transformed cells are a case in point (Casto, 1972; Burns and Black, 1969; Dunn et al., 1973).

The relationship between transformation and virus DNA replication is unclear in the sense that in some transformed cells the virus DNA

appears to replicate or be induced to do so (Watkins, 1973; Klein, 1975; Andersson, 1975); while in others there is no evidence to suggest that normally it does (Green, 1970; McDougall et al., 1975; McDougall, 1974).

This last point is important since the failure of the virus DNA to replicate suggests that a very basic level of restriction exists in the transformed or tumour state. The amount of virus DNA in transformed or tumour cells is an indication of this and is therefore an important feature of virus transformation or tumourogenesis.

AMOUNT OF VIRUS DNA IN TRANSFORMED OR TUMOUR CELLS

Determination of the amount of virus DNA within transformed or tumour cells is important for a few reasons. As mentioned previously an absence of large amounts of virus DNA per cell would suggest that virus DNA replication - outside mitosis - is unlikely to be a feature of or a prerequisite for transformation or tumourogenesis. But estimation of the exact amount, or nearest approximation to this, is equally important because it may reflect a more basic feature of either the transformation or oncogenic process. For instance, it could be argued that the frequency of transformation of an individual cell is dependent on the frequency of virus DNA molecules within it: the more virus DNA molecules the more likely will the cell become transformed. Alternatively the presence of only one virus DNA molecule per cell, for example, might indicate that the cell's tolerance is low, either at the gross or the molecular level. Examples would be the failure of the cell to remain viable with more than a few virus DNA molecules or, at the molecular level, the presence of only one potential integration site for the virus DNA.

While it is clearly desirable to obtain this information, attempts to do so have resulted, as will now be summarised, in different estimates for the amount of virus DNA in certain virus transformed or tumour cells.

The number of copies of virus DNA within transformed or tumour cells as been studied by making use of molecular nucleic acid hybridisation and reassociation techniques: principally, RNA excess hybridisation using radioactive complementary (cRNA) to the virus DNA; and reassociation of radioactive virus DNA in conjunction with unlabelled transformed or tumour DNA.

In the former type of approach the amount of virus DNA is estimated usually in conjunction with reconstruction experiments using pure virus DNA. That is, the amount of cRNA hybridised to a particular amount of virus DNA is compared with the amount bound to transformed or tumour DNA. Using this method, a variety of virus-transformed cells and tumours have been studied (Table 1:1). What is evident from these studies is the fact that several copies of virus DNA appear to exist in several transformed cell types. Thus Green (1970) estimated 60 copies of Adenovirus DNA in an Adenovirus 12 transformed cell line; 85 in an Adenovirus 7 transformed cell line; and 23-29 in an Adenovirus 2 transformed cell line. And SV40 transformed cells appear to possess 5-60 virus DNA copies (Westphal and Dulbecco, 1968) while several human lymphoblastoid cell lines possess around 20-100 EBV DNA copies (Pagano, 1974; Klein, 1975; Nonoyama and Pagano, 1971; Zur Hausen et al., 1972).

The principle of the second method of virus DNA estimation (Also Table 1:1) is that a precisely defined amount of labelled virus

DNA is allowed to renature after denaturation, the reaction following characteristic kinetics dependent on the initial DNA concentration and its complexity (Britten and Kohne, 1966; Wetmur and Davidson, 1968; Kohne and Britten, 1971). Various cellular DNAs can then by analysed for the presence of virus DNA by adding them to the renaturing virus DNA and observing the deflection and increase in its reaction rate. Using this technique, Gelb et al., (1971) detected between 1 and 3 SV40 genome copies per diploid quantity of SV40 transformed cell DNA; and Smith et al., (1972) demonstrated a similar low amount of SV40 DNA in abortively-transformed BALB/3T3 clones. Small amounts of virus DNA sequences in SV40 transformed cells have also been reported by Ozanne et al (1973) where five independently-derived transformed clones contained 1.35-8.75 copies per diploid quantity of host DNA. For Adenovirus transformed or tumour cells the amount of virus DNA, as determined by this technique, is also low: thus one Adenovirus transformed rat cell line contains close to one virus DNA copy (Pettersson and Sambrook, 1973).

There is therefore some discrepency between the results obtained by RNA excess hybridisation and virus DNA-DNA reassociation. This is exemplified in the case of the Adenovirus 2 transformed cell line, 8617. By cRNA hybridisation the virus genome number estimates are between 14 and 30 (Green, 1970: Green et al., 1970) whereas by the virus DNA-DNA reassociation technique there is approximately only one copy which is detectable (Pettersson and Sambrook, 1973). These discrepencies mean, in effect, that the facts supporting hypotheses on the role of virus DNA in transformed or tumour cells are themselves controversial.

(a)

Table 1:1

Virus	Transformed or Tumour cell	No. of virus genome copies (or equivalents) /diploid quantity host DNA	cRNA-DNA hybridisation	virus DNA-DNA reassociation	virus DNA-host DNA reassociation	Reference	Comments
EBV	"Raji"	50	+	-	-	Zurhausen et al.(1970)	
	HKLY-1(nc)	26	+	-	-	"	nc = Nasopharyngeal Carcinoma
	HKLY-2(nc)	20	+	-	-	"	
	D75(BBrTR)	188	+	-	-	"	
	BL	26	-	-	+	Zurhausen et al(1970)	BL = African Burkitt's lymphoma
	B2	22	-	-	+	"	
	nc	19	-	-	+	"	
	nc	6	-	-	+	"	
	Raji	60	+	-	-	Glaser & Nonoyama('72)	
	Raji	6	-	-	+	Zurhausen & Schult-Holthansen (1970)	labelled virus DNA double-stranded and of low specific activity
	Raji	57	+	-	-	Nonoyama & Pagano (1971)	
	Raji	50.8-52	-	+	-	Nonoyama & Pagano (1973)	

Table 1:1 contd.

Virus	Transformed or Tumour cell	No. of virus genome copies (or equivalents) /diploid quantity host DNA	cRNA-DNA hybridisation	virus DNA-DNA reassociation	virus DNA-host DNA reassociation	Reference	Comments
SV40	SVT2	1.52	-	+	-	Gelb et al.(1971)	
	SV40 hamster tumour	2.08	-	+	-	"	
	SV-UV-15(5)	3.86	-	+	-	"	
	SVpy3T3/1	1.42	-	+	-	"	
	SV-UV-15(1)	1.04	-	+	-	"	
	SVT2	1.56	-	+	-	"	
	SVT2	1.56	-	+	-	Gelb & Martin(1972)	
	SV-UV-15(1)	1.04	-	+	-	"	

(b)

Table 1:1 contd.

Virus	Transformed or Tumour cell	No. of virus genome copies (or equivalents) /diploid quantity host DNA	cRNA-DNA hybridisation	virus DNA-DNA reassociation	virus DNA-host DNA reassociation	Reference	Comments
SV40	SV3T3	8-9	-	+	-	Ozanne et al(1973)	the quantity of 3.9 x 10^{12} daltons for host DNA used for calculations
	SVT2	2.2	-	+	-	"	
	SV101	8-9	-	+	-	"	
	SVB30	6.1	-	+	-	"	
	SVpy11	1.3	-	+	-	"	
	F1SV101	8-9	-	+	-	"	
	CA41.6	8-9	-	+	-	"	
	CA30.4	8-9	-	+	-	"	
	CA32.6	8-9	-	+	-	"	
	SV3T3-47	20	+	-	-	Westphal & Dulbecco (1968)	
	SVPy3T3-11	44	+	-	-	"	doubly infected cell (polyoma and SV40)
	H50	58	+	-	-	"	
	SV3T3	20	+	-	-	Sambrook et al(1968)	

Virus	Transformed or Tumour cell	No. of virus genome copies (or equivalents) /diploid quantity host DNA	cRNA-DNA hybridisation	virus DNA-DNA reassociation	virus DNA-host DNA reassociation	Reference	Comments
Adeno-virus	Ad2/F2 (50pfu/cell) Ad2/B8 (10fu/cell)	50-150	+	-	-	Dunn et al(1973)	In situ hybridisation estimates
	Ad2/8617	0.98-1.04 0.79-1.00	-	+	-	Pettersson & Sambrook(1973)	
	Ad2/8617	2.7	+	-	-	Loni & Green(1973)	In situ hybridisation estimates
	Ad7/5728	10.7	+	-	-	"	"
	Ad12/HE/9	5.5	+	-	-	"	"

(c)

Table 1:1 contd.

Virus	Transformed or Tumour cell	No. of virus genome copies (or equivalents) /diploid quantity host DNA	cRNA-DNA hybridisation	virus DNA-DNA reassociation	virus DNA-host DNA reassociation	Reference	Comments
Adeno-virus	Ad2	23-29	+	-	-	Green(1970)	
	Ad7	85	+	-	-	"	
	Ad12	22	+	-	-	"	
	Ad12 hamster	22	+	-	-	Green et al(1970)	
	Ad12 transformed	53-60	+	-	-	"	
	Ad7 hamster tumour	86-97	+	-	-	"	
	Ad2(8617)	22-30	+	-	-	"	
	Ad2(8629)	29	+	-	-	"	
	Ad2(8638)	14	+	-	-	"	
	Ad2(8625	37	+	-	-	"	
Poly-oma	Py3T3-6	5	+	-	-	Westphal & Dulbecco(1968)	
	Py8	7	+	-	-	"	
	Svpy3T3-11	10	+	-	-	"	Doubly infected cell (polyoma and SV40)

Table 1:2

(a) Human Adenoviruses

Particle weight $x10^6$ daltons	175
Diameter of virion mμ	80
% DNA	12-13
DNA, m.w., $x10^6$ daltons	20-25
DNA conformation	linear duplex
No. of polypeptides	9
Host cell for productive infection	human
Host cell for transformation	hamster, rat, human

(b) Human Adenoviruses

Group	Members	Oncogenicity	%DNA	Viral DNA % G+C
A	Ad12,18,31	"highly oncogenic" in newborn hamsters	11.6-12.5	48-49
B	Ad3,7,11, 14,16,21	"weakly oncogenic" in newborn hamsters (all but Ad11)	12.5-13.7	49-52
C	Ad1,2,5,6	"non-oncogenic" in newborn hamsters but morphologically transform rat embryo cells in vitro	12.5-13.7	57-59

(a) Taken from Green (1970).

Some of the discrepencies do appear to be due to technical factors (e.g. Haas et al., 1972) although it is unlikely that they can all be explained in this way (for a fuller discussion see Chapter III, Discussion pgs. 129).

Work described in this Thesis was undertaken with a view to measuring and locating Adenovirus DNA in particular transformed or tumour cells by various techniques in order to attempt to settle questions of the sort outlined this far. The basic methodology employed was cRNA-DNA hybridisation. Its advantages are as follows: ease of synthesis of highly radioactively-labelled complementary RNA to the virus DNA template; the detectibility limits involved with molecular cRNA-DNA hybridisation can be large; and cRNA -DNA hybridisation in situ is possible allowing single cells to be studied, potentially to determine cytological localisation of viral nucleic acids.

CHOICE OF ADENOVIRUS SYSTEM

For technical reasons the Adenovirus system was easier to study than some other mammalian DNA virus systems: Adenoviruses can be readily grown in Human Embryonic Kidney (HEK) or Hela cells which greatly facilitates their isolation; the virus genome is relatively large ($\sim$20-25 x 10^6 daltons) facilitating detection and isolation; and Adenovirus stocks, tumours and transformed cells could be obtained (see Materials and Methods). They are indigenous to man (Pereira et al., 1963; Ginsberg, 1962); and as previously mentioned the human serotypes differ in oncogenic potential (Trentin et al., 1962; Green, 1970; Huebner, 1967). The basic features of human Adenoviruses are presented in Table 1:11.

INTEGRATION OF VIRUS DNA WITHIN CELLULAR DNA OF TRANSFORMED AND TUMOUR CELLS

Although quantitative estimates of virus DNA in many different virus-transformed or tumour cells vary, estimates for individual cell lines are constant even through many cell generations. For instance, the "Raji" human cell line derived from an African Burkitt's lymphoma always possesses in the region of 50 EBV genome copies even after several periods of cloning (Klein, 1975; Pagano, 1974). This does suggest that virus DNA replication, outside mitosis, is unlikely to be a feature of these cells; and that the virus DNA may be associated with the host cell DNA: possibly integrated with it. If this is generally the case then an important feature of transformation or tumourogenesis is likely to be virus DNA associations with chromosomes which could provide the means by which the virus DNA in transformed cells, for example, is not only stably inherited (Green, 1970; Marin and Littlefield, 1968; Marin and Macpherson, 1969), but influences and is influenced by the control mechanisms inherent in their structure. Some of the evidence for viral integration with host cell DNA will now be discussed.

a) Association of virus DNA with cellular DNA

Infective native SV40 DNA exists as a superhelical twisted circular molecule. These structures are found in productively infected cells (Sebring et al., 1971). However, they are not found in SV40 transformed cells (Sambrook et al., 1968; Westphal and Dulbecco, 1968). In addition, by applying a DNA extraction method which separates infective virus DNA from cell DNA (Hirt, 1967), the above authors showed that SV3T3-transformed cells do not possess free virus DNA in detectable amounts either. Chromosomes, however,

possess the same amount of SV40 DNA sequences as total nuclear DNA; and alkali-stable covalent linkages exist between the virus DNA and the host DNA which suggests that virus DNA becomes covalently integrated into the chromosomal DNA in these transformed cells.

Covalent linkage of virus DNA sequences to host cell DNA sequences has also been suggested by alkaline CsCl gradient centrifugation followed by SV40 cRNA hybridisation (Collins and Sauer, 1972). These authors showed, moreover, that virus integration appears to be independent of cell DNA synthesis, a finding complemented by Doerfler (1970) who demonstrated that integration of Adenovirus 12 into Baby Hamster Kidney cellular DNA was independent of host DNA synthesis.

For EBV DNA in non-producer Raji cells, this does not appear to be the case since a large percentage of virus DNA is separated from host DNA on alkaline glycerol gradients (Nonoyama and Pagano, 1972). This, however, does not preclude the possibilities that the virus and cellular DNAs are associated through alkali-labile bonds; that a small percentage of the EBV DNA is associated in alkali-stable bonds; that EBV DNA contains nicks or single strand interruptions that are alkali-labile; a strong possibility since another Herpes virus, Herpes Simplex, is susceptible to alkali treatment (Frankel and Roizman, 1972) and may possess ribonucleotide regions (Biswal et al., 1974). By way of clarification, Adams et al (1973) and Adams and Lindahl (1975) have recently shown that a small percentage of EBV does appear to integrate with host cell DNA, while the rest of it is separable from host DNA.

Very much less is known about the Adenovirus group although Green (1970) has suggested that Adenovirus DNA is integrated into host cell DNA in several Adenovirus transformed cell lines since DNAs isolated from chromosomes and nuclei have the same virus DNA content. These are unpublished results however and remain to be confirmed.

The difficulties with all these experiments lie first of all with the detectibility limits and secondly with the fact that advantitious DNA binding can never be ruled out completely. Thus failure to detect virus DNA in certain DNA fractions might be due to technical limitations and co-migration of virus DNA with high-molecular weight DNA might, in some cases at least, reflect non-specific DNA-DNA interactions.

b) Association with host cell chromosomes

1. Association of virus-specific functions and chromosomes.

Somatic cell hybrids can be formed by fusing mammalian DNA virus transformed or tumour cells with normal cells (Glaser and O'Neill, 1972; Glaser and Nonoyama, 1972; Glaser and Rapp, 1972; Weiss et al., 1968; Marin and Littlefield, 1968; Weber, 1974; Huenber and Kowprowski, 1974; Klein et al., 1974). Three reports in particular, have a direct bearing on the persistence of virus DNA within cells and its association with host cell chromosomes.

In the first report, two variants of BHK21 cells were used: one lacking inosinic acid pyrophosphorylase (IPP) and resistant to 6-thioguanine, the other lacking thymidine kinase and resistant to 5-bromodeoxyuridine (Marin and Littlefield, 1968). A hybrid was obtained which was sensitive to both analogues and had twice the

normal BHK chromosome number. After transformation with polyoma virus, an IPP-deficient subline was isolated. This hybrid possessed transformed properties. However selection for resistance to either analogue resulted in chromosome loss. In 6-thioguanine resistant clones, a loss of chromosomes also resulted in a proportion of cells which possessed normal cell characteristics, for example reduced plating efficiency in agar. Two of these clones also lost the polyoma T-antigen and were less tumourogenic than related transformed cell lines. Upon reinfection however these clones became transformed and polyoma T-antigen became re-detectable (Marin and Macpherson, 1969). These results were interpreted to indicate that selection for loss of the chromosome(s) controlling the synthesis of IPP brought about, in a few cases, the loss of chromosomal factors controlling the transformed phenotype. In the "revertant" clones with "normal" phenotype, about 20% of the chromosomes of the hybrid had been lost. This suggests that the polyoma genome, or at least some virus-mediated controlling function, is associated with one or a few chromosomes in the karyotype. This is a slightly different conclusion from that of a conceptually similar experiment carried out by Weiss *et al.* (1968) who demonstrated that hybrid cells formed between SV40 transformed human cells and normal mouse cells synthesised SV40 T-antigen; but upon extended cultivation and after nearly *all* human chromosomes had been lost, this synthesis was no longer detectable. Virus or virus-mediated gene expression therefore appears to be correlated with the presence of several host cell chromosomes in this case. Klein *et al.* (1974) have also shown that EBV-determined nuclear antigen (EBNA) and other EBV-associated antigens are not synthesised

in hybrid clones - originally derived from the fusion of an EBV DNA positive lymphoblastoid cell line with a mouse cell line - which have lost several human chromosomes. They conclude therefore that while EBV DNA may be associated with several human chromosomes, it is not associated with them all.

Conclusions derived from the above experiments, while generally valid, may not be valid in detail however; particularly in view of the fact that the authors failed to identify the chromosomes unambiguously. Association of T-antigen expression, for example, with several chromosomes might be a misinterpretation since specific chromosomes might be retained preferentially. For the SV40 transformed human cell - normal mouse cell hybrids studied by Weiss et al. (1968) this does appear to be the case since there is preferential retention of human chromosome 7 which is also the only one to be consistently associated with SV40 T-antigen expression (Croce et al., 1973). Virus gene-expression may therefore be associated with only one or two chromosomes in other hybrid cells also (e.g. Klein et al., 1974).

Virus T-antigen expression and its association with chromosomes of the transformed or tumour cell has also been suggested by chromosome transfer techniques. Metaphase chromosomes isolated from an inducible SV40 transformed Chinese hamster cell line, with no detectable infectious virus or free virus DNA, on transfer to permissive BSC 1 cells, bring about the appearance of SV40 T-antigen in one cell per 10^4 treated cells (Shani et al., 1974). This is unlikely to be due to transfer of T-antigen alone since activation of the antigen in the permissive cells is wholely dependent on the integrity of the chromosomes during transfer.

2. Virus-chromosome interactions.

Viruses can have visible effects on host cell chromosomes of subsequently transformed cells. These effects can be random or non-random. Thus, early after infection of hamster cells with Adenovirus 12 or 2 there is a gradual appearance of stable chromosome aberrations in the subsequently transformed cells (Stich and Yohn, 1970); and some of these cells possess new marker chromosomes. Many other transformed cells also possess chromosome aberrations and breakage is frequently a feature of the original exposure of the cells to virus (e.g. see Jones, 1974; McDougall, 1975). That chromosome breakage may reflect virus integration is suggested by a few findings. During productive infection, for example, chromosome breakage has been reported (McDougall, 1971; Zur Hausen, 1967; Cooper et al., 1967; Stich and Yong, 1967) and integration also appears to be a regular feature of productive infection. However, chromosome aberrations and breakage in transformed cells can also be caused by agents other than viruses: for example X-rays (Caspersson et al., 1972), inhibitors of DNA synthesis (Benedict et al., 1970) which act during the cell's G2 or S phase (Karon and Benedict, 1972), and chemical carcinogens (Nichols, 1966; DiPaolo et al., 1973). However the fact that certain DNA inhibitors appear to be incorporated into host DNA while they cause chromosome breakage (Karon and Benedict, 1972) and the finding that X-rayed cells are more prone to transformation by DNA viruses (Stocker, 1963) as are cells with spontaneous chromosome aberrations (Todaro et al., 1966; Swift and Hirshhorn, 1966) suggests that virus integration may occur via chromosome breakage. If this is so, then virus release (during rescue with

permissive cells for example; or during induction with agents such as mitomycin C or BUDR) may occur via chromosome breakage also. This is supported by the findings that induction of virus in some transformed cells by DNA analogues is accompanied by the chromosome breakage. Furthermore, it has been demonstrated that amino acid deprivation can result both in chromosome breakage (Freed and Schatz, 1969) and induction of infectious SV40 from transformed hamster cells (Kaplan et al., 1972). There is therefore a tentative correlation between integrative mechanisms and chromosome breakage.

3. Association of virus DNA with chromosomes.

In the non-lytic infection of hamster cells (BHK 21 and NIL) with ^{3}H-Thymidine-labelled Adenovirus 12, association of grains with chromosomes was observed (Zur Hausen, 1968). The label was in grain clusters which were absent from non-inducible rat kangaroo cells infected with the same virus, thus suggesting that the association of grain clusters with chromosomes represented integration of virus DNA. After UV irradiation (Zur Hausen, 1968) which reduces the infectivity of the virus in permissive cells, this chromosome association was still observed suggesting that integration is not affected by UV exposure. Since, in several cases, transformation is resistant to UV irradiation (Latarjet et al., 1967) whereas virus assembly or infectivity appears to be affected, the inability of UV to affect the association of virus DNA label with chromosomes can be explained on the basis that initiation of transformation and virus DNA integration are correlated. In line with this is the finding that even early virus functions, such as virus DNA replication, are post-integrative (Doerfler, 1968).

By studying the early infection of cultured human leukocytes, which are non-permissive, with ^{3}H-Adenovirus 12, Nichols et al. (1968) found a random association of grains with host cell chromosomes.

However neither the experiments of Zur Hausen (1968) nor Nichols (1968) can rule out the possibility that the label associated with host chromosomes represents binding of virus DNA to host chromatin. To this extent they are somewhat inconclusive.

4. The use of in situ hybridisation.

Non-specificity can largely be circumvented by the application of the in situ hybridisation method (see Chapter 111, section 1V). The advent of this technique (John et al., 1969; Gall and Pardue, 1969) with its inherent molecular and cytological specificity, has enabled the precise chromosomal mapping of specific nucleic acid sequences. Correspondingly it has been utilised in attempts to detect virus-specific DNA sequences associated with host cell chromosomes. Thus attempts have been made to localise EBV DNA (Zur Hausen and Schulte-Holthasen, 1972); SV40 DNA (Oda et al., 1972); and Adenovirus DNA (McDougall et al., 1972b; Dunn et al., 1973; Loni and Green, 1973; Green, 1970) within transformed or tumour karyotypes.

The results of such attempts tend to suggest a random distribution of virus DNA throughout individual karyotypes. However it is unclear whether some of these results are wholely interpretable on this basis. Some pointers suggest they might not be. First, the evidence from somatic cell hybridisation studies in general suggest a less wide distribution of virus DNA in various karyotypes. Second, in some cases, and especially where there is no distinct chromosomal location,

the results of in situ hybridisation experiments are inconclusive (see Jones and Bishop, 1973 for example).

Part of the difficulty in interpretating in situ hybridisation results of this kind lies in the nature of the technique itself and our limited knowledge of its efficiency. Thus although conventional molecular hybridisation techniques such as DNA excess or RNA excess hybridisation can be characterised with respect to their reaction parameters (also see pg. 74) it is more difficult to do this for in situ hybridisation reactions because of the complications arising from the fact that the DNA "targets" are embedded in the chromosome. Reflecting this difficulty, very few studies have commented on how the actual process of in situ hybridisation compares with other nucleic acid hybridisation techniques. Nevertheless, with these reservations, in situ hybridisation can be a very useful technique in the present context because of its inherent specificity. However, because even the largest estimates of virus DNA in transformed or tumour cells are relatively low (Table 1:1) and because of some uncertainty in previous results (see above) it was considered important to determine whether individual in situ hybridisation reactions behave as conventional hybridisation reactions which can be optimised, thus increasing the chances of detecting virus DNA These experiments are described in Chaper 111, section IV. Experiments involving in situ hybridisation under optimal conditions to cells containing virus DNA are described in Chaper 111, section V.

SUMMARY

The persistence of virus DNA is a general feature of virus-transformed or tumour cells. Although for a variety of virus-transformed or tumour cells the amount of virus DNA varies, it is clear that it can be subject to replicative and transcriptional controls (also see Chapter IV) which themselves are likely to be influenced by both virus and host cell. The actual virus DNA amount in individual cell lines or tumours is particularly important to estimate since it could reflect a basic feature of the transformation or oncogenic process. Furthermore, it is evident that the virus DNA has associations with chromosomal and/or cellular DNA sequences. This, as well, may have important implications for both transformation and oncogenesis. From this point of view, *in situ* hybridisation possesses great potential. The following chapter deals with the points raised here.

ABBREVIATIONS

DNA	deoxyribonucleic acid
RNA	ribonucleic acid
HnRNA	heterogenous nuclear RNA
rRNA	ribosomal RNA
rDNA	ribosomal DNA
mRNA	messenger RNA
DNase	deoxyribonuclease
RNase panacreatic (A)	ribonuclease from bovine pancreas
poly A	poly-adenylic acid
poly U	poly-uridylic acid
oligo dT	oligo-thymidylate
EDTA	ethylenediamine tetra-acetic acid sodium salt
SLS	sodium lauryl sulphate
SSC	standard saline citrate
TRIS	trishydroxymethylamino-methane
TCA	trichloroacetic acid
PCA	perchloric acid
PPO	2-5-diphenyloxazole
POPOP	1,4, bis-2-(4-methyl-5-phenyoxazole)-benzene
MEM	minimal essential medium
BSA	bovine serum albumin
GF filters	glass fibre filters
DEP	diethyl pyrocarbonate
HEK	human embryonic kidney
O.D.	optical density

m.o.i.	multiplicity of infection
S.C.	sub-cutaneous
EBV	Epstein-Barr Virus
F.A.	Formamide
p.f.u.	plaque forming units
UDP	Uridine diphosphate
cpm	counts per minute (radioactivity)
G.C.	Guanine + Cytosine content
S.A.	Specific activity
cRNA	complementary RNA
Ad or Adeno-	Adenovirus
SV40	Simian Virus 40
Py	polyoma virus
cDNA	complementary DNA
UTP	Uridine triphosphate
ATP	Adenosine triphosphate
GTP	Guanosine triphosphate
CTP	Cytidine triphosphate
FBS	Foetal Bovine serum
FCS	Foetal Calf Serum
DFCS	Dialysed FCS

BUFFERS AND SOLUTIONS

RSB	10mM TRIS pH7.5
	10mM NaCl
	1.5mM $MgCl_2$
SSC	150mM NaCl
	15mM Na citrate
Column Buffer	0.3M NaCl
	0.01M Na acetate
Binding Buffer	0.4M NaCl
	10mM TRIS pH7.5
	1mM EDTA
	0.1% SLS
Eluting Buffer	10mM TRIS pH7.5
	1mM EDTA
	0.1% SLS
"Kirby" solution	1% Na-triisopropylnaphalene Sulphonate
	6% Na-4-amino salicilate
	1% NaCl
	6%n-Butanol
	0.5% SLS
Phenol-cresol	500gm phenol=550ml water-saturated phenol
	70gm m-cresol
	0.5gm 8-hydroxyquinoline
Trypsin	Made up to a 0.25% solution in Dulbecco A and sterilised through Millipore filters (HAWP, 0.22 u pore size)

Dulbecco A/

	200mg/litre $K_2H_2PO_4$
Dulbecco A	$8x10^3$mg/litre NaCl
	200mg/litre KCl
	1,150mg/litre Na_2HPO_4
PBS	200:1 Dulbecco A: Dulbecco B
	(Dulbecco B=$CaCl_2$ 100mg/litre
	$MgCl_2$ 100mg/litre)
TryptosePhosphate Broth (TPB)	29.5gm Difco Bacto TPB in 1 litre
	of distilled water

Counting Fluids

Toluene-PPO-POPOP	3gm PPO and 0.3mg POPOP per litre
	of toluene
Aquasol	NEN chemicals

CHAPTER II (MATERIALS AND METHODS)

MATERIALS

a) Cells

Human Embryonic Kidney cells were obtained from Foetuses donated by Dr. Brock, Western General Hospital, Edinburgh.

Adenovirus 2 transformed cells were donated by Dr. P. H. Gallimore, Dept. Cancer, Birmingham. The lines were as follows:

Ad2/REB/10p/BI Rat embryo brain cells infected at a mo.i. of 10pfu/cell.

Ad2/REB/50p/BI Rat embryo brain cells infected at a mo.i. of 50pfu/cell.

Adenovirus 7 transformed hamster cells were obtained from Flow laboratories, England: cat. no. TT-103.

b) Tissues

1. Human placental tissue was obtained for the preparation of human DNA from the Simpson Maternity Pavilion in Edinburgh.

2. Tumours or cells derived from tumours were obtained as follows:

Ad2/HL REF/50p/T5, or Ad2/T5 for short, was induced in new born Hooded Lister (HL) rats by inoculating Adenovirus 2 transformed cells (fibroblasts) originally infected at 50pfu/cell into them without immunosupression. (Not the same as Ad2/REB/50p/BI)

Ad2/HL REB/50p/T6, or Ad2/T6 for short, was induced in new born Hooded Lister rats by inoculating Adenovirus 2 transformed cells originally infected at 50pfu/cell into them without immunosupression. (Not the same as Ad2/REB/50p/BI or cells inducing Ad2/T5.)

Ad2/T4 was a tumour induced by the inoculation of Adenovirus 2 transformed cells into newborn Hooded Lister rats under conditions of immunosupression. In this case the original transformed cells were infected at a m.o.i. of 10pfu/cell and are identical to the Ad2/REB/10p/BI/

Ad2/REB/IOp/BI line described above.

Ad12/TI was an Adenovirus 12 tumour induced by innoculating purified Adenovirus 12 virus into newborn Hooded Lister rats at an infectivity of $2x10^8$ - $2x10^9$ p.f.u. in HEK cells. No immunosupression.

Immunosupression is necessary in certain cases of tumour induction by the "non-oncogenic" Adenoviruses (see Table 1.2) (Gallimore, 1972; 1974).

3. Viruses

In the main, three Adenoviruses, each one belonging to a different serotype group (see Table 1.2), were used in these studies: Adenovirus 2, Adenovirus 7, and Adenovirus 12. All original stocks of these viruses were a gift from Dr. J. K. McDougall, Dept. Cancer Studies, Birmingham. In addition to these viruses, Adenovirus 5, which was a gift from Dr. J. Williams, MRC Virology Unit, Glasgow, was also used in certain experiments.

Unless otherwise stated, all chemicals were obtained from British Drug Houses (B.D.H.).

METHODS

The methods section is divided into subsections which correspond roughly to the different sections of the subsequent CHAPTERS III and IV. Methods are arranged essentially in order of their appearance in the Thesis.

SECTION 1

1. Culture Media for cells

a) HEK cells were grown and maintained in F10 medium (Ham, 1963) supplemented with 10%tryptose phosphate broth (DIFCO Labs.) and 10% FCS or 10% FBS. (Biocult Ltd., Glasgow).

b) Hela cells were grown in Eagle's MEM (Hanks based plus non-essential amino acids), supplemented with 0.1% $NaHCO_3$, 0.13mg/ml streptomycin, 60ug/ml penicillin, and 10% FBS. Normal mouse cells, rat cells and transformed cells were also grown in this medium.

c) Eagle's MEMS plus 5% FCS or DFCS was used in transformation experiments. (Gallimore, 1974).

d) Tumour oells, derived from induced tumours, were grown in Eagle's MEMS which was supplemented with 10% FCS, sodium pyruvate, and 2xamino acids plus vitamins.

2. Culture Preparation of Tumour Cells

Tumours were excised from the surrounding rat tissue and were macerated with sterile scissors. The macerate was washed with Dulbecco A (Oxoid Ltd., London) and the cells suspended in Dulbecco A without the need for trypsin. The cells were then pelleted by low speed centrifugation for 2-3 minutes, and resuspended in Dulbecco A. The cell suspension was agitated and a sample counted to ascertain the number of cells present. MEMS (supplemented) was warmed

to/

to 37°C and added to the cell suspension. After addition of antibiotics, the cell suspension plus new medium was added to litre, sterile burler bottles. Cells were grown at 37°C, with the medium being changed every three days.

3. Extraction and Preparation of DNA from whole tissues

Modification of the method of Marmur (1961) was used. The procedure is described in Prosser (1974). Tissues were cut up, minced and placed into saline EDTA at 0°C and washed several times to remove any blood. The connective tissue of tumours, or placenta, was removed and the remaining tissue homogenised in a loose-fitting glass Teflon homogenizer (approximately 5ml saline EDTA to each gram of tissue). SLS was added to a final concentration of 2%, and the mixture incubated at 60°C for 10 minutes. Sodium perchlorate (5M) was added to a final concentration of 1M. Then a 1/10 of a volume of chloroform-isoamyl alcohol (24/1:v/v): phenol was added and the solution agitated for about $\frac{1}{2}$ hour. After shaking, the material was centrifuged at 10K rpm (12,000G) for 10-20 minutes in the Sorval (RC2 B) at 4°C. The top layer was withdrawn with an inverted pipette and transferred to a beaker. Two volumes of absolute ethanol were carefully over-layered on the DNA solution. The crude DNA was washed through an alcohol series, air dried and finally dissolved in 0.1xSSC. After dissolving, 1/10 volume of 10xSSC was added followed by solid NaCl to a concentration of 1M. RNase (Sigma) in 2% Na acetate pH5.0, (concentration: 20mg/ml) was heated to 100°C for 5 minutes, chilled to room temperature and added to the DNA solution to a final concentration of 50ug/ml. If the starting tissue was liver, an amylase (Sigma) digestion step wa included at a concentration of 200ug/ml. This enzyme was added at the same time as the RNase. The solution was incubated/

incubated for 3-4 hours at 37°C. Protease (Sigma) was then added to a final concentration of 400ug/ml, and the digestion carried out for up to 6 hours at 37°C. The solution was then shaken with chloroform-isoamyl alcohol:phenol, 1:1, and centrifuged as before. The aqueous layer was removed and the deproteinising step was repeated until no protein interphase was observed between the two layers. In order to precipitate the DNA from the solution, acetate EDTA (3M Na acetate, 0.001M EDTA), pH7.0, was added (1/10 volume) followed by isopropanol (0.56 volume). The DNA was spooled out of solution, dehydrated through an alcohol series, air dried, and dissolved in the desired solvent (e.g. 0.1xSSC).

4.4 Extraction and Preparation of DNA from tissue culture cells

Cells were grown to confluence, or nearly so, in Petri dishes or in 1 litre burler bottles. They were then washed several times in Dulbecco A to remove culture medium. Trypsin (0.25% in Dulbecco A) was added and the cells incubated at 37°C. Detached cells were centrifuged in an MSE bench centrifuge at 5K (approx. 2,300 rpm) for 5 minutes. The supernatant was removed and the cells washed again with Dulbecco A. After a second centrifugation, DNA was extracted either by the modified Marmur method (see above) or as follows. The pellet was carefully resuspended in a small amount of residual supernatant. Protease (Sigma) was made up in 2xSSC (200ug/ml), digested at 37°C for 30 minutes, adjusted to 0.1% SLS, and added to the cells suspension which was incubated at 37°C for 2-3 hours. 4ml aliquots of this solution were then made up with 5.12 gm CsCl (BDH, analytically pure) and centrifuged for 40 hours at 40K rpm at 25°C. Fractions were collected, diluted with 0.1xSSC, and their optical densities (260nm) determined. The fractions containing the

DNA/

DNA peak were pooled and pelleted for 18 hours at 30K rpm in the 3x20 swing out rotor of the MSE 50 ultracentrifuge. The DNA was redissolved in 0.1xSSC or other required solvent.

5. Preparation of Micrococcus luteus DNA

10gms of M.Luteus cells (Sigma) were mixed with 100mls of 0.01M Tris pH8.0 at room temperature in an MSE blender for 1 minute. The washed cells were pelleted in the Sorval for 10 minutes at 10K rpm. The cells were resuspended in 0.01M Tris, 0,2M sucrose pH8.0 and the solution made up to 200mls. 75mg of lysozyme were made up in 1.5ml Tris-sucrose buffer and the solution heated to 30°C. This lysozyme solution was then added to the M.Luteus solution and the mixture heated at 30°C for 15 minutes, 0.25ml of 0.01M $MgCl_2$ were added and the mixture left for 30-40 minutes. During this time 100ml of 0.45M NaCl, 0.3M EDTA pH8.0 were made up and to this solution, 12ml of 25% SLS were added. This solution was then heated to 60°C. The heated solution was now added to the M. Luteus solution after the 30 minute incubation with $MgCl_2$. The mixture was mixed thoroughly. After lysis, 78mls of 5M $NaClO_4$ and 40mls saturated Tris pH8.3 were added. The DNA was then deproteinised by shaking with phenol-chloroform, 1:1; precipitated, and purified by the method for making DNA from whole tissues.

6. The presence of contaminating RNA was checked by alkaline digestion of a sample of DNA. Approximately 50ug/ml of DNA was treated with 1/10 vol. of 6N KOH at 37°C for 1 hour. 1/10 vol. of concentrated P.C.A. was added and the sample then left on ice for approximately 20 minutes. The sample was centrifuged at 10,000 rpm for 10 minutes to precipitate the DNA and the potassium perchlorate. The optical density (260nm) of the supernatant was determined after careful decanting. Only DNA samples that were uncontaminated with RNA were used/.

used. Usually, after alkaline digestion and acid precipitation of the DNA sample, the supernatant O.D. was negligible.

7. Growth of Adenovirus in cell cultures

Adenovirus was passaged in human embryonic kidney cells (HEK) or Hela cells. Cells were infected at virus multiplicities of 50-100pfu/cell. After usually 48 hours, the infected cells were disrupted by ultrasonic treatment and 2gm. of CsCl (B.D.H., analytically pure) were added to approximately 4ml. aliquots of virus/cell extract. The samples were then centrifuged to equilibrium in a SW65 rotor in a Beckman L2-65B ultracentrifuge. (Russell et al.; 1967). After several centrifugations in CsCl the virus band was dialysed against 0.01M Tris pH7.2 for up to two days at 4°C. The virus particles were left for a further three days in the Tris buffer and the DNA extracted after this time.

8. Purification of Adenovirus DNA (Levine and Ginsberg, 1967)

The DNA was extracted from virus particles by a modification of the procedure of Borenfreund et al. (1961). The virions were disrupted by 1% SLS incubation at 4°C for 30 minutes. 0.25M 2-mercaptoethanol was added and the mixture shaken for 30 minutes at 4°C. Pronase (1mg/ml) was added and the solution shaken for a further 60 minutes at 37°C. Two volumes of chloroform-isoamylalcohol (19:1) were added and 1 vol. of this mixture was added to 1 vol. of water-saturated phenol. The mixture was shaken for 10 minutes at room temperature. After centrifugation at 10,000 rpm for 10 minutes, the aqueous phase was removed and re-extracted. The aqueous phase, after the second centrifugation, was removed and the DNA precipitated with 2 volumes of absolute alcohol. The precipitated DNA was then centrifuged at 10,000 rpm for 10 minutes, the supernatant discarded, and the precipitate redissolved in about 1ml of 3mM NaCl. The dissolved DNA

was/

was re-precipitated with alcohol and the precipitate again centrifuged.

9. Analytical centrifugation and determination of initial buoyant densities

Centrifugation was carried out in the Beckman Model E Analytical Ultracentrifuge for 18 hours at 44K rpm at 25°C. Ultraviolet photographs were taken and traced on the Joyce Loebel microdensitometer. The densities of the DNAs in neutral CsCl were determined from the position of a marker DNA added to the gradients. (Usually M.Luteus DNA, 1.731gm/cm^{-3}).

0.89gm CsCl (B.D.H. analytically pure) were added to 0.7ml DNA solution (0.1xSSC) containing 5ug DNA and the density was brought to 1.710gm/cm^{-3} (10°10') by refractometry and according to the relationship:
p^{25}_{25} = 10.8601 Nd^{25}-13.4974 (11ft, Voet, and Vinograd, 1961) where p is the buoyant density at 25°C, Nd^{25} is the Refractive index at 25°C.

Analytical runs were usually carried out by Mr. I. F. Purdom, (this lab)

10. Thermal Denaturation of DNAs

Denaturation was carried out in a Unicam SP800 Spectrophotometer. Variable and constant temperatures were achieved with the Unicam temperature programmer (SP876) and heating block (SP877) attachments. Readings of optical absorbance were taken on a linear recorder (SP20) via a scale expander (SP850). Unicam microcells (10mm path length and 0.45ml volume) were used to hold the DNA samples. These cells were held in the heating block and the holders fitted with a clamping device to seal the cells and prevent evaporation. The sample of DNA, and a reference sample, were degassed by taking the samples up in a 1ml syringe, blocking the needle with a rubber stopper, creating a vacuum in the syringe, and tapping out the air bubbles. The temperature of the heating block was raised at 1°C/minute until the hyperchromicity reached a plateau. The time was recorded on a print-out chart. The percentage increase/

increase in hyperchromicity was plotted against temperature (°C). DNAs were denatured in 1mM EDTA (Spiers, unpublished), or in 1xSSC.

11. Single stranded molecular weight determination

The method of Studier (1961) was employed. Equal volumes of DNA (200ug/ml) in 0.1xSSC and 0.2M NaOH in 0.1xSSC were mixed and left for 2 hours at room temperature. The alkaline DNA solution (20ul) was sedimented through 0.9M NaCl, 0.1M NaOH (700ul) at constant velocity. The single stranded molecular weight was determined from the $_{20}S_w$ of the DNA according to the formula:

$$\text{Molecular Weight (M.W.)} = \log^{-1}\left(\frac{\log {}_{20}S_w + 1.05453}{0.346}\right)$$

, Allowance was made for temperature and salt concentration as described (Studier, 1961). Mr. J. Telford or Mr. I. Purdom performed the molecular weight determinations.

12. DNA renaturation

DNA was purified and checked for RNA contamination. Only DNA in which there was negligible contamination by cold alkali soluble material was used. Aliquots (15mls.0.1xSSC) were sonicated for 15" pulses with intervals of 2 minutes between pulses to a total of 1'30" sonication with a Dawe sonicator (position 8). After sonication, the DNA was precipitated by the addition of 2 volumes of absolute alcohol and 1/10 volume of 2M sodium acetate pH5.0 and left at -20°C for a minimum period of 2 hours. The DNA was collected by centrifugation at 10,000 rpm for 15 minutes in the HB4 rotor of the Sorvall. The pellet was dissolved in 0.3M NaCl, 0.01M sodium acetate (Column buffer). The solution was loaded onto a 2.5cmx45cm column of Sephadex SE50, swollen in the same buffer. The DNA was eluted with column buffer and the optical densities of each fraction determined. The peak fractions were pooled/

pooled and precipitated with 2 volumes of absolute aclohol and stored at -20°C. The precipitate was collected by centrifugation and the DNA dissolved in the desired solvent (e.g. 0.1xSSC). For renaturation, the concentration of the DNA was generally 10-20mg/ml.

The average molecular size of the sonicated DNA was in the region of 10^5 daltons (Alkali single strand).

DNA renaturation was generally carried out at 65°C or 70°C in 2xSSC (Bishop, 1972). The DNA, dissolved in 0.1xSSC pH5.5, was denatured by heating in a boiling water bath for 7 minutes. A control sample was withdrawn and diluted in 0.1xSSC pH7.5 (ice-cold). The rest of the DNA solution was transferred to a water bath at the required temperature of incubation, and the solution left for 30 minutes to allow the temperature to equilibrate. 20xSSC pH5.0 was added to the solution to a final salt concentration of 2xSSC. After thorough mixing, zero time points were taken and diluted in to ice-cold 0.1xSSC. The liquid paraffin was layered on the top of the DNA solution and the reaction tube was stoppered to avoid evaporation. Samples were withdrawn at different times and diluted into ice-cold 0.1xSSC. The final concentration of the DNA in the 0.1xSSC was around 50ug/ml. Duplicate samples for each point were examined in theUnicam SP800 spectrophotometer and the absorbance of each sample was recorded from 320-230 nm at 50°C. The temperature was then raised to 90°C to melt the duplexes, and the spectra of the samples again recorded. The rise in extinction, at 260nmm, between 50°C and 90°C, is due to the hyperchromicity of the double stranded DNA and is therefore a measure of the amount of renaturation which has taken place. It is therefore possible to plot:

$$\frac{E260\ (90) - E260\ (50)}{E260\ (90)}$$ as a measure of renaturation.

Fully/

Fully denatured DNA has 138% of the E260 of native DNA. From the E260 (90) it is therefore possible to calculate the E260 native value:

$$\text{E260 native} = \frac{\text{E260 (90) x100}}{138}$$

The amount of renaturation which has taken place at a given Cot value can thus be expressed as a percentage of the total possible renaturation by:

$$\text{Renaturation} = \frac{\text{E260 (90) - E260 (50) x 100}}{\text{E260 (90) - } \dfrac{\text{E260 (90) x 100}}{138}}$$

The percentage of renatured DNA can be plotted against Cot:DNA initial concentration in moles nucleotide/litre^{-1} times time in seconds (Britten and Kohne, 1968).

SECTION II

1. In Vitro Transcription

a) Standard incubation mix: All glassware was washed in chromic acid, distilled water, autoclaved overnight at 110°C, and then siliconised. Radioactive nucleotides were obtained from the Radiochemical Centre, Amersham, and unlabelled ones from Sigma. E.Coli DNA-dependent RNA polymerase was obtained from Sigma or Miles-Seravac. In some experiments enzyme prepared by Dr. J. O. Bishop (this laboratory) was used. M.Luteus DNA-dependent RNA polymerase was obtained from Miles-Seravac. Unless otherwise stated in the text or independent Figure legends, the transcription mix was standard. With all four nucleotides labelled (usually with specific activities: ATP, 20 Ci/mmole; UTP, 14 Ci/mmole; CTP, 20.8; GTP, 10 Ci/mmole), this standard mix was as follows: 0.1M Tris pH7.5, 0.5mM $MnCl_2$, 4mM $MgCl_2$, 1.6mM Spermidine, 70mM -100mM KCl, 2mM K_2HPO_4, 5nmoles each of ^{3}H ATP, ^{3}HGTP, ^{3}HUGT, ^{3}HCTP, and finally 2.5 units of enzyme. The final volume was 0.1ml, and in addition to the above reagents, it contained 1-5ug of high molecular weight native DNA.

For preliminary experiments this DNA was lyophilised in a 0.001M NaCl solution prior to addition of the incubation mix. Sometimes, however, this lyophilised step was found to have serious effects on the commencement and rate of the transcription reaction after addition of the enzyme. This was probably due to the delay in the DNA coming into solution. Consequently, for the majority of the transcription reactions, the DNA was previously concentrated to the desired amount in 0.001M NaCl before being added to a previously made up reaction mix. Under these conditions the reaction commenced immediately the enzyme was added (Figure II:I).

To/

Figure II:I.Transcription of Adenovirus 2DNA by the E.coli DNA-dependent RNA polymerase with the DNA(Iug) lyophilised(o-o) or added in a 0.00IM NaCl solution(x-x)(see text). The incubation mix contained IOOmM Tris pH7.5, 2mM K_2HPO_4,0.IM KCl,0.0005M $MnCl_2$,0.004M $MgCl_2$,2.5 units enzyme,5 nmoles each of ATP,GTP,CTP,UTP(all tritium labelled:I5-20Ci/mmole) and the volume was made up to 0.Iml with distilled water.Incubation was at 37°C,points being taken at intervals during the reaction.The aliquots were TCA precipitated(IO%) and the radioactivity determined by counting in Toluene-based scintillation fluid.

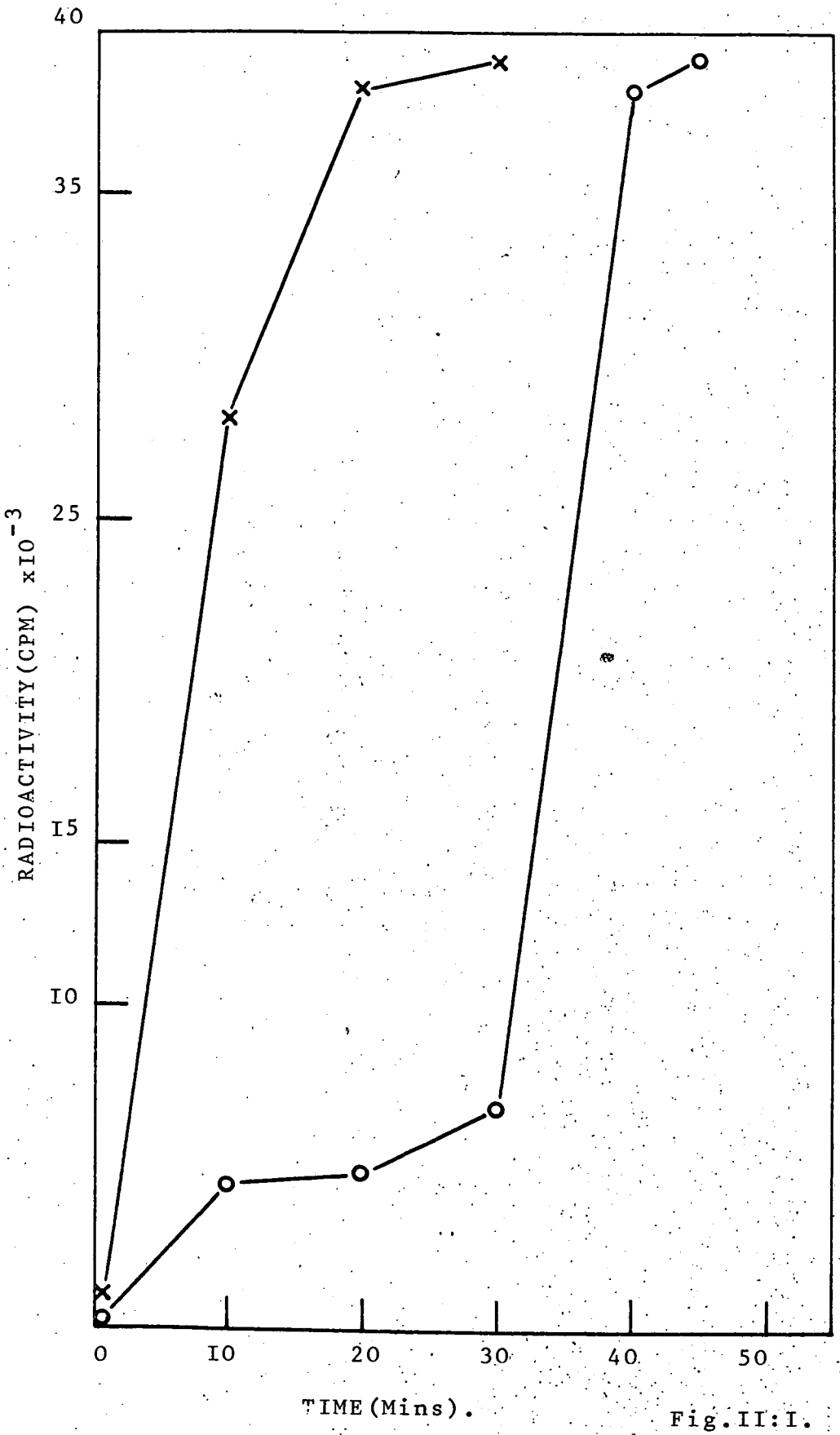


Fig.II:I.

To monitor the incorporation of the radioactive triphosphates into RNA, 2ul. samples were withdrawn from the incubation mix at certain times and the amount of RNA determined by TCA precipitation. Zero time points were withdrawn before the enzyme was added to the mix. The transcription reaction was carried out at 37°C and when the incorporation reached a plateau, the reaction was terminated by chilling on ice. The method used here has been utilised by Jones et al. (1974).

b) Extraction and Purification of cRNA

cRNA was prepared in the above manner or as varied in the text or Legend Figures. DNase was added to the chilled transcription mixture to a final concentration of 40ug/ml (4ul of a 1mg/ml solution made up in 0.01M Tris pH7.5, 0,002M $MgCl_2$ and 10% dimethylsulphoxide and stored at -20°C) and the mixture incubated for 10 minutes at 37°C. Then 10ul of 1M NaCl and 10ul of 5%SLS were added and the mixture incubated for a further 2 minutes at 37°C. Approximately 150-200ug of carrier unlabelled E.Coli RNA were added and the RNAs extracted with water saturated distilled phenol (BDH, analar). After vigorous shaking, and centrifugation to separate the aqueous and phenol layers, the phenol layer was re-extracted with an equal volume of 0.1xSSC. The combined aqueous phases were placed directly on a Sephadex G-50 (Pharmacie Fine Chemicals, Uppsala) column (30cmx1.5cm) previously equilibrated with 0.1xSSC. The RNAs were eluted in 0.1xSSC at a rate of 1ml/5 minutes, and the fractions collected on an LKB fraction collector controller, type 3403 B. The RNA peak was located by spectrophotometric reading at 260nm, and the radioactivity of the cRNA monitored by TCA precipitation and counting in Toluene-based scintillation fluid. The principal fractions containing the cRNA were pooled, lyophilised, and the RNA resuspended in the desired solvent./

solvent. In some experiments the pooled RNA fractions were made 0.3M NaCl and the RNA precipitated with 2.5 volumes of absolute alcohol. After overnight precipitation at -20°C the RNA was pelleted and resuspended in the desired solvent. The specific activity of cRNAs, using all four ribonucleoside triphosphates labelled, was estimated to be 1.4 - 2×10^7 cpm/ug.

2. Separation of E.coli total RNA

E.coli M.R.E.6 00 (MRC Microbial products Division, Porton, Wilts) were broken up and dispersed in 40mls of cold 0.01M $MgCl_2$, 0.01M sodium azide, and 0.01M Tris. The cells were centrifuged at 10,000 rpm for 10 minutes and the pellet ground with 4gms of Al_2O_3 for 10 minutes. 12ml of Tris/$MgCl_2$ buffer were added and the mixture centrifuged at 10,000 rpm for 10 minutes. After re-centrifugation of the supernatant, DNase (20ug/ml final) was added and the solution incubated for 2 minutes at 37°C. The incubatedsupernatant was chilled and centrifuged at 38,000 rpm for 60 minutes at 0°C. The resulting pellet was homogenised in 5.0ml of Tris/$MgCl_2$ and then centrifuged at 15,000 rpm for 10 minutes. The supernatant was made 0.5% SLS and an equal volume of phenol was added. After shaking for 10 minutes the aqueous and phenol phases were separated by centrifugation. The aqueous and proteic-phases were re-extracted with phenol. To the final aqueous phase 1/10 a volume of 2.0M potassium acetate pH5.0 was added, followed by 2 volumes of ethanol. The RNA was precipitatedovernight at -20°C. Total RNA was stored in alcohol.

3. Fractionation of unlabelled E.coli RNA into 23S and 16S RNA

E.coli total RNA, which was precipitated in absolute alcohol, was pelleted by centrifigation in the Sorval SS-34 rotor for 10 minutes at 10,000 rpm. The pellet was then washed at least once with cold alcohol and then dissolved in 0.001M EDTA, 0.1M NaCl, 0.1% diethyl-pyrocarbonate/

pyrocarbonate (Kodak) and 0.02M Sodium acetate ph5.0. The RNA was layered on to a 5-40% Sucrose gradient, the sucrose being dissolved in the same buffer. These RNA gradients were centrifuged at 25,000 rpm for 18 hours at 10-15°C in the 6x15 MSE rotor. Fractions were collected from the bottom of each tube after piercing with a needle. The optical density (260nm) of each fraction was determined in the SP800 spectrophotometer and the 23S and 16S peaks pooled and precipitated with 2 volumes of absolute alcohol after adding 1/10 the volume of 3.0M NaCl. The RNA was precipitated overnight at -20°C; collected by centrifugation in the Sorval SS-34 rotor and was washed again with absolute alcohol. After pelleting once more the RNA was dissolved in the appropriate salt and frozen at -20°C.

4. Fractionation of labelled cRNA in a linear Sucrose gradient

An appropriate amount of cRNA was dissolved in 0.001M EDTA, 0.01M NaCl, 0.1% DEP and 0.02M sodium acetate pH5.0. This solution, together with a solution of E.coli ribosomal RNA dissolved in the same buffer, was gently layered on to a pre-made 5-40% sucrose linear gradient. Centrifugation was carried out as described for the fractionation of E.coli RNA, and the optical densities of each fraction determined. TCA precipitable radioactivity was measured for fraction aliquots, and the distribution of cRNA molecules along the gradient analysed with respect to the unlabelled E.coli 23 and 16S marker RNAs.

SECTION III

1. Pancreatic RNase (Sigma)

The enzyme was made up in 2% sodium acetate pH5.0 and heated at 100°C for 5 minutes before being diluted into 2xSSC at approximate concentrations.

2. DNase (Sigma)

10mg of electrophoretically pure DNase were suspended in 2ml of 0.0025N HCl and dialysed for 2 days against 2 litres of 0.0025 N HCl. 2.5ml of 0.2M sodium acetate pH5.3 and 0.75ml of 1M sodium iodoacetate were added to this DNase solution. The mix was incubated at 55°C for 60 minutes, and then dialysed overnight against 1 litre of 0.0025 N HCl.

The precipitate was spun down at 10,000 rpm for 30 minutes and the supernatant then contained DNase at the approximate concentration of 2mg/ml.

3. Filter Hybridisation procedure

Denatured DNA was loaded onto membrane filters (13 Millipore, HAWP 0.45µm pore size) according to the method of Gillespie and Spiegelman (1965). DNA, usually in 0.1xSSC, was denatured by the addition of an equal volume of 1N NaOH for 15 minutes at room temperature. The solution was then neutralised with 2 volumes of neutralising mix (1.0N HCl, 1.0M Tris pH8.0., 3.0M NaCl, 1:1:2 by volume) and allowed to drip through the membrane filters which had been prewashed in 2xSSC. Loaded filters, and blanks, were washed with 6xSSC, and subsequently dried in a vacuum oven at 80°C for 2 hours. They were then labelled with a pencil and stored at -20°C

4. RNA-DNA hybridisation in RNA excess

Before hybridisation, the filters containing bound DNA were soaked in/

in the reaction medium minus RNA. Temperature optimums (T.OPT.) for individual hybridisation reactions were determined by the method of Birnstiel et al.(1972), the reaction usually not exceeding 30% of the saturation value. For kinetic studies, the hybridisation medium was brought to optimal temperature, filters introduced and individual ones withdrawn and placed into chilled 6xSSC at various times over approximately 10-80% of the reaction. Filters were washed by the batch method (Birnstiel et al.; 1968), an RNase step being included. Controls consisted of heterologous DNA alone, and blank filters. RNA-DNA hybrids were counted in Toluene-based scintillation fluid.

In experiments involving hybridisation of cRNA across a CsCl or a Ag^{+}_{-} Cs_2SO_4 gradient, fractions, or aliquots from fractions, were loaded onto millipore filters and hybridised as above so that the amount of cRNA in the reaction would always be in excess of the homologous DNA sequences.

5. DNA-RNA hybridisation in DNA excess

The reactions were carried out according to the procedure of Melli et al. (1971) and Bishop (1972a). The concentrations of the DNA and RNA werechosen so that there was usually a 100:1 or 1000:1 excess ratio of DNA:RNA. Under these conditions the reaction is likely to be complete (Bishop, 1972b) although in some cases the ratio had to be adjusted to allow for a specific base sequence ratio. In general DNA was present as 10-20mg/ml. and the RNA was present in very small amounts. The actual concentrations of the RNA was dependent on both the DNA base sequence:RNA base sequence ratio, and the specific activity of the labelled RNA. For cRNA the specific activity was $1.4\text{-}2.0\times10^{7}$cpm/ug. When the reaction was considered to be incomplete the concentration of DNA in the mixture was increased: i.e. the

DNA/

DNA:RNA ratio was increased.

Purified, sonicated DNA was denatured in 0.1xSSC by heating at 100°C for 7-10 minutes. RNA was added and the mixture heated for a further 3-4 minutes. A control sample was taken and diluted into ice-cold 2xSSC. The DNA and RNA mixture was removed to a constant temperature water bath, (65°C or 70°C). The hybridisation reaction was started by adjusting the solution concentration to 2xSSC. Zero time points were taken and the reaction monitored by sampling for various times. Each sample was diluted into ice-cold 2xSSC. To determine the amount of hybrid formation throughout the reaction, samples were divided into equal volumes, and the RNA-DNA hybrids detected by RNasing (10ug/ml panacreatic; Sigma) followed by TCA precipitation, alcohol rinsing, drying in a vacuum oven at 80°C, and finally counting in Toluene-based scintillation fluid. The % RNase-resistance for each sample was obtained by comparing RNased samples with non-RNased samples. This % RNase-resistance was then plotted against log Cot (Melli et al., 1971; Bishop, 1072; Bishop 1972b; Campo, 1973).
For DNA excess hybridisation reactions which were continued for several hours, or even days, 2% SLS was added to the original reaction solution. In these cases, samples were diluted in 2xSSC so that the final SLS concentration was 0.005% or less since higher concentrations interfere with RNase digestion.

6. Preparation of labelled Xenopus ribosomal RNA

Cells of a Xenopus permanent cell line were grown to 2/3 confluence in a 250ml Falcon flask. They were labelled with 80uCi/ml. ^{3}H-Uridine (>20Ci/mmole, Amersham) in Eagle's MEM, supplemented with 0.1% $NaHCO_3$, 10%FBS, and antibiotics; and incubated for 48 hours at 25°C. The cells were washed with Dulbecco A and homogenised at 0°C in 0.15M NaCl, 0.1% SLS, 0.01M Tris pH7.2. An equal volume of water-saturated phenol

was/

was added and the RNA purified by repeated phenol extraction and centrifugation. The final aqueous phase was made 2% sodium acetate (pH5.0) and the RNA precipitated by adding 2.5 volumes of absolute alcohol. The precipitate was stored at -20°C overnight. 28 and 18S RNAs were separated by centrifugation in a linear 5-40% sucrose gradient as described for the isolation of E.coli 23 and 16S RNAs. The specific activity was 2×10^5 cpm/ug.

7. Preparative CsCl gradient centrifugation

The method of isopycnic centrifugation has been reviewed by Flamm et al. (1967; 1969). DNA in 0.1xSSC, together with M.luteus DNA as a density marker (1.731gm/cm^{-3}), was added to 5.2gmCsCl (BDH, analytically pure) and centrifuged in an MSE 10x10 rotor for 40 hours at 25°C, running speed 42Krpm. A hole was pierced and fractions collected. After diluting to 0.5ml with 0.1xSSC, the optical density of each fraction was determined.

8. Self-Complementarity of labelled RNA preparations

RNA was denatured by heating at 100°C for 5 minutes. After rapid chilling in ice, the RNA was incubated in an SSC or an SSC/FA mix at appropriate temperatures of incubation (see text or Legends for details). At various times during the annealing reaction, samples were withdrawn and diluted into ice-cold 0.1xSSC (or 2xSSC). After the last sample had been diluted, they were split into two equal portions. One lot was RNased (10ug/ml pancreatic; Sigma) at 37°C for 30 minutes; the other was incubated at the same temperature and for the same time with 0.1xSSC. Carrier RNA (E.coli total, or yeast; (Sigma)) was added and the RNAs precipitated by adding an equal volume of 10% cold TCA. After 20 minutes on the ice samples were filtered on GF/C filters. The collected precipitate was washed twice with cold 5%TCA followed by one rinse in absolute/

absolute alcohol. Filters were then dried in a vacuum oven at 80°C for approximately 20 minutes. 10ml of Toluene PPO-POPOP were added and the samples were counted in a Packard scintillation counter.

9. Dissociation of RNA-DNA hybrids (membrane filters)

cRNA-DNA hybrids were formed at optimal rate temperatures in appropriate hybridisation solutions, and the reaction terminated at approximately 80% of the final saturation value. To determine the temperature at which 50% of the cRNA was released from each filter (Tm), the filters were placed in 1.0ml of melting solution (generally 1xSSC) after nuclease inhibition, and heated in temperature increments of ~8°C (Birnstiel et al. 1972). The details of the procedure are as follows: The RNA-DNA hybrids, on filters, were counted in Toluene-based scintillation fluid. To remove the Toluene fluid the filters were washed in chloroform (3 changes) for a total of 15 minutes. After drying, the filters were soaked in 1XSSC containing 0.1% diethylpyrocarbonate and then washed for 1/2 hour in this solution at room temperature. These filters were then washed in 1XSSC at room temperature for a further 20 minutes. Each filter was then placed in a vial and 1ml. of melting solution added. After 5 minutes at each temperature of incubation the 1ml. was removed from the vial and set aside on ice. Another 1ml. of solution was added to the vial containing the filter and the temperature of the solution raised by approximately 8°C. Samples were taken, in this fashion, until the temperature approached 100°C. 200ug carrier RNA was added to each 1ml stored in ice. 50% cold TCA was added to make the RNA solution 10%TCA, the tubes stirred, and left in ice for 20 minutes. The RNA was then filtered through GF/C filters which were washed with 5% cold TCA, rinsed with absolute alcohol, and dried in a vacuum oven at 80°C for 20-30 minutes. The dried filters were then counted in 15ml of Toluene-based scintillation fluid. In addition, the original/

original membrane filter was also counted after drying. The % TCA precipitable RNA released from the membrane filter was plotted against the temperature increase. All the RNA, originally in hybrid form, was generally recovered.

10. Preparation of labelled Adenovirus 2 DNA

Human embryonic kidney cells (HEK) were grown at 37°C. The culture medium was Ham's F10 plus 10% tryptose phosphate broth plus 10% FCS. Adenovirus 2 (a gift from Dr. J. K. McDougall, Birmingham) was added to the cells and was absorbed for 3 hours in medium without the serum content. After this time the cells were rinsed in PBS and the medium plus the 10% FCS replaced. Infected cells were incubated at 37°C for 10 hours before ^{3}H-Thymidine (20Ci/mmole; Amersham) was added at a concentration of 1uCi/ml. At this time virus-specific DNA is beginning to be synthesised (Ledinko and Fong, 1969; Dunn et al., 1973). Cells were harvested after 70 hours incubation in medium plus ^{3}H-Thymidine and virus extracted and purified according to the previously described procedure. Virus DNA was extracted according to the method of Levine and Ginsberg (1967) which is described above. The DNA had a buoyant density of 1.716gm/cm^{-3} in neutral CsCl, both in the preparative ultracentrifuge and in the analytical ultracentrifuge. Two preparations of ^{3}H-Thymidine labelled Adenovirus 2 DNA were used; one batch being prepared by Dr. J. K. McDougall, Birmingham. Both preparations possessed specific activities of 10^{6}cpm/ug.

SECTION IV

1. Preparation of chromosome spreads from cultured cells

Cells were grown to $\frac{1}{2}$-$\frac{2}{3}$ confluence in 9cm Petri dishes (Sterilin, Flow Laboratories, Ayrshire) in 10ml of culture medium, generally Eagle's MEM supplemented with 10% FCS and containing 0.1% $NaHCO_3$, and antibiotics. Colcemid (Ciba) was made up 1mg in 100ml. Dulbecco A (OXoid) heated to 37°C. 0.4ml were added to each Petri dish which contained 10mls medium. The colcemid was left to act on the cells for 5-6 hours of culture at 37°C. The medium was then pipetted off and the cells washed carefully with Dulbecco A. Pre-warmed Trypsin (0.25% in Dulbecco A) was added to the cultures and the dishes rocked gently until the cells began to detach from the substrate. These cells were pelleted for 5 minutes in an MSE bench centrifuge set at 2K (1400 rpm). The supernatant was removed and the cells suspended in fresh Dulbecco A. After thorough rinsing and shaking in the Dulbecco A, the cells were again pelleted as before. The supernatant was decanted and the pellet resuspended in a very small volume of residual supernatant Dulbecco A. Care was taken to completely resuspend the cells at this juncture. Approximately 5ml of 0.07M KCl were slowly run into the centrifuge tube containing the cells. The tube was agitated and the cells suspended and dispersed in the KCl solution which was then warmed at 37°C for 10-15 minutes. The cells were again pelleted, the KCl removed, and the pellet resuspended in residual drop of supernatant. Cells were then fixed in 3:1 methanol:acetic acid. Freshly prepared 3:1 fixative was run into the tube and the cells dispersed. They were then left for at least one hour. The fixative was changed 3 times, the cells being pelleted between each change. Fixed cells were dropped onto previously cleaned glass microscope slides (boiled in concentrated HCl, rinsed in alcohol-ether and thoroughly washed in running/

running distilled water) and the spots air-dried.

Alternatively the pellet was fixed in 2.5% glutaraldehyde (TAAB Lab., Reading) in 0.1M phosphate buffer, pH7.2 for 30 minutes at room temperature. Cells were then washed repeatedly in the phosphate buffer and dropped onto glass slides and left to air dry.

2. Preparation of chromosome spreads from whole blood

Peripheral blood was drawn by venipuncture into a disposable plastic syringe and immediately transferred to a plastic lithium heparin bottle (Stayne Laboratories, High Wycombe) and thoroughly mixed. Universal bottles (10ml) were prepared containing 4ml culture medium (Eagle's MEM + 0.1% w/w $NaHCO_3$, 10%FCS, and antibiotics) and 0.05m; phytohaemagglutinin (Burroughs Wellcome). 0.4ml of blood was incubated at 37°C for 2-3 days. During the last 3 hours of incubation, 0.5ml of 0.02% colcemid solution (Ciba) made up in Dulbecco A was added. The cultures were gently shaken and then spun down on an MSE bench centrifuge at 5 for 5 minutes. The supernatant was discarded and the cells resuspended in pre-warmed (37°C) 0.07M KCl. The rest of the procedure was been described in the preparation of chromosome spreads from cultured cells, (see pg.44).

Blood was donated by Dr. J. Prosser and Mr. N. Thomas (both this laboratory) and was withdrawn by Dr. R. Sutcliffe (Dept. Genetics, Glasgow).

3. Cells grown on microscope slides

Clean, sterile galss microscope slides were prepared and placed in Petri dishes. Culture medium was added to the dishes, and an appropriate dilution of cells, in the same medium, added. The cells were incubated at 37°C in an atmosphere of 5% CO_2 in air. The medium was washed from the slides and the cells rinsed in Dulbecco A. After

several rinses in the Dulbecco A the cells were swollen in 0.075M KCl; and fixed in 3:1 methanol:acetic acid (cold) or in 2.5% Gluteraldehyde as described previously. After several changes in fresh fixative, the slides were left in fixative in the cold (4°C) overnight. They were then passed through an alcohol series (50-100%) and left to air-dry.

4. In situ hybridisation

a) Cytological preparations:

1. Cell cultures: Chromosome spreads and interphase nuclei were prepared as described.
2. 5um frozen sections of tumour material were prepared by Dr. J. K. McDougall, Birmingham.

b) The hybridisation reaction:

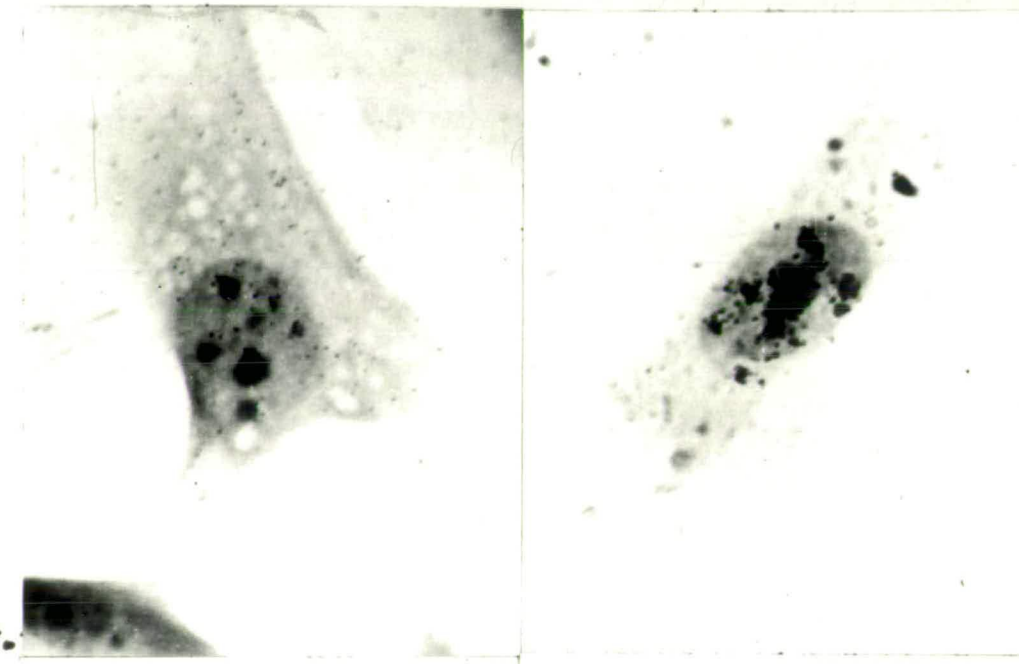
Coverslips (Chance, no.1, 22mmx22mm) were cut to 11x11mm size, washed in alcohol: ether 1:1, siliconized (Repelcote, Hopkins and Williams, Chadwell Heath), dried, rinsed in distilled water and dried again. A rubber sealing solution was prepared by mixing Cow Gum (P.B. Cow Ltd., Slough, Bucks) with petroleum ether (B.D.H.).

The fixative used has an effect on the results obtained after in situ hybridisation. Figure 11:2 and Table 11:1 show that with 3:1 methanol: acetic acid as opposed to gluteraldehyde the final outcome in terms of grain counts is better. For most in situ hybridisation experiments described here the fixative was therefore always 3:1 methanol:acetic acid.

The denaturation method also has an effect on the results of in situ hybridisation experiments. Gall and Padue (1969) have used NaOH to denature the chromosomal DNA. However, both AHNSTROM and Natarajan (1974) and Commings et al.(1973) have shown that there is considerable loss of DNA from the chromosomes during the NaOH treatment. Heat

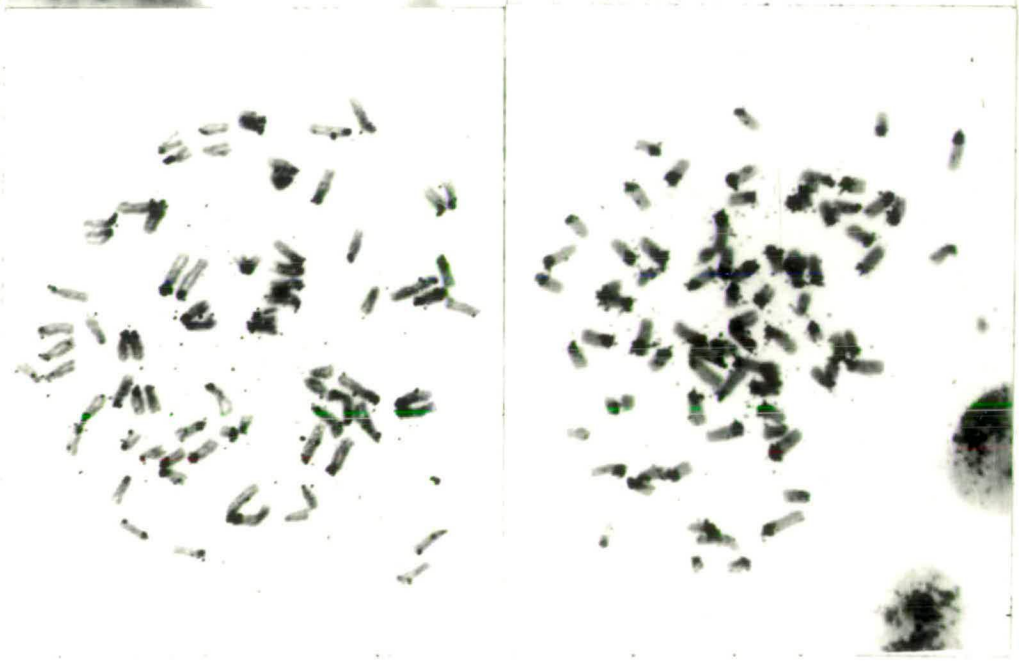
denaturation/

Figure II:2. Mouse embryo cells after *in situ* hybridisation with mouse satellite cRNA. Conditions of hybridisation: I hour, 3xSSC, 60°C, 0.001ug /3ul cRNA. Stained in Giemsa (pH 6.8). a) whole embryo cells fixed with 2.5% Gluteraldehyde: exposure time 2 weeks; b) whole embryo cells fixed with 3:I methanol:acetic acid: exposure time 2 weeks; c) mouse chromosomes fixed in 2.5% Gluteraldehyde: exposure time 6 weeks; d) mouse chromosomes fixed in 3:I methanol:acetic acid: exposure time 6 weeks. a) and b) x400 in scale; c) and d) x450. Note the higher grain yield in methanol:acetic acid fixed preparations.



a.

b.



c.

d.

Table II:1

	*% Cells containing n grains in nucleus					Grains in cytoplasm (Average background)
Fixative	n= 5	6-10	11-25	26-50	51	
2.5% Gluteraldehyde	0	26	60	14	0	10-20
Methanol:acetic acid, 3:1	0	0	9	21	70	5

Conditions of hybridisation as for Figure II.2. Exposure time 2 weeks.

* No. of cells counted = 100 for both fixatives.

denaturation at high temperatures has also been used (John et al.1969) as has heat denaturation at low temperatures in conjunction with Formamide (Steffensen and Wimber, 1970) which reduces the melting temperature of DNA duplexes (Helmkamp and Ts'o, 1961 and McConaughy et al. 1969).

Figure II:3, however, shows that there also appears to be loss of DNA from the chromosomes with heat treatment in conjunction with Formamide. No loss appears to occur with HCl treatment which has also been used to denature chromosomal DNA (MacGregor and Kezer, 1971, Jones 1973). In addition, NaOH and heat Formamide treatment appeared to have deleterious effects on chromosome morphology and stainability with giemsa. Therefore unless otherwise stated HCl denaturation was always used in the in situ hybridisation experiments in this thesis.

Acid denatured cytological preparations were freed of dust particles by blowing them with air. Radioactive cRNA was applied to the preparations in the appropriate salt solution (usually 2-4xSSC) and a coverslip sealed to the preparation with the diluted Cow Gum. Slides were incubated usually at the optimal rate temperature of hybridisation for each cRNA-DNA reaction. The reaction was terminated after several $t\frac{1}{2}$s for individual hybridisations and the coverslip removed and the slides dipped into ice-cold 2xSSC. These slides were washed in cold 2xSSC for several minutes, then RNased (20ug/ml in 2xSSC) for 20-30 minutes in 2xSSC at 37°C. After RNase digestion preparations were washed exhaustively in a large volume of 2xSSC in the cold (4°C). 3-4 hours later and after repeated changes of 2xSSC, the preparations were dehydrated through an alcohol series: 50-100%, and subsequently air-dried at room temperature.

c) Autoradiography/

Figure II:3. Loss of DNA on treatment with various denaturants used in the *in situ* hybridisation procedure. Mouse embryo cells were labelled with ^{3}H-Thymidine for approximately two cell generations and nuclear and chromosome spreads made. These spreads were then subjected to DNA denaturants and the amount of incorporated ^{3}H-Thymidine remaining examined by autoradioagraphy. Exposure time: 2 weeks.

^{3}H-Thymidine labelled cells treated with a) 0.07N NaOH for 3mins. at R.T.^{o}C; b) 0.2 N HCL for 20mins. at R.T.^{o}C; c) 0.IxSSC 90%F.A. for 2 hours at 65^{o}C.

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c) Autoradiography

Ilford K2 or Ilford L4 Nuclear Emulsion in gel form was used to coat slides for autoradiography. A 1:1 solution of emulsion:distilled water was heated to 39-43°C in a water bath. The mixture was gently stirred to avoid local overheating, to mix the emulsion with the water thoroughly, and to produce an even surface for the dipping of the slides. Dipped slides were hung vertically and dried in a stream of air at 18°C. They were then stored in light proof boxes at 4°C for varying lengths of time. Slides were developed in Kodak D 19 B developer for 3.5 minutes with no agitation, at 18°C, rinsed carefully in distilled water, fixed in Johnson Fix-Sol (diluted 1:5 with distilled water) for 5.5 minutes (or 2x the clearing time), washed with distilled water for several minutes and stained.

d) Staining Autoradiographs

Slides were stained in Giemsa R66 (Gurrs, London) diluted 3/100 in Buffer pH6.8 (Gurr's tablets). The staining time was generally around 1/2 hour - 1 hour depending on the thickness of the emulsion film. Overstained preparations were destained in either Buffer pH6.8 or 50% alcohol.

5. In situ hybridisation involving cells grown directly on glass slides, and cells derived from blood cultures

This was carried out as described previously (pg.46). Cells grown on glass slides were always denatured with HCl; NaOH and heating removed the cells. HCl-denatured cells were not removed during either the fixation procedure or the subsequent in situ hybridisation procedure.

6. Alkaline CsCl gradients

These gradients were prepared according to the protocol of Flamm et al. (1967) with the inclusion of M.Luteus DNA, alkaline buoyant density 1.788 gm/cm^{-3} (Vinograd et al.,(1963). A trace amount of highly purified/

purified satellite DNA was added to 3.3mls of a 0.01M Tris-HCl (pH8.5) solution containing 40ug Xenopus DNA (a gift from Mr. C. Philips, this laboratory) and 20ug M.Luteus:DNA:100ul IN NaOH were added, followed by 500ug SLS. The solution was finally brought to an initial density of 1.760gm/cm^{-3} with CsCl (B.D.H., analytically pure) and the DNA centrifuged in the MSE 50 rotor for 40 hours at 44,000 rpm at 25°C. The buoyant densities of the separated strands of the satellite DNA were calculated by comparing their positions in the alkaline gradient with those of the M.Luteus and Xenopus DNAs. These marker DNAs have buoyant densities in alkaline CsCl of 1.788gm/cm^{-3} and 1.754gm/cm^{-3} respectively. (The values were determined in the analytical Ultracentrifuge).

7. Preparation of Satellite DNA

Satellite and main band DNA were separated by preparative density centrifugation in Ag^+ - Cs_2SO_4 (Jensen and Davidson, 1966). The Technique relies on the selective affinity of Ag^+ - ion for certain DNA bases and has been used to isolate a variety of satellite DNAs from a variety of organisms; for example, from the Chimpanzee (Prosser et al., 1973); man (Corneo et al., 1973); Baboon (Prosser, 1974) and mouse (Corneo et al., 1968) Here, mouse satellite DNA was purified by the method of Corneo et al.(1968) and Human satellite DNAs, I, II, and III prepared by the methods of Corneo et al.(1970; 1971).

Purified total DNA was dissolved in 0.1M Na_2SO_4 and then dialysed against 0.1M Na_2SO_4. A solution of 0.1M borate buffer pH9.2 was added to give a final concentration of 0.005M borate ion. A 10^{-3} M solution of $AgCLO_4$ was added to give varying molar ratios of Ag^+ to DNA-Phosphate. For the mouse, R^F values of 0.2-0.25 were used: for the human satellites I, II, and III, R^F values of 0.1, 0.35 and 0.2 were used. A saturated solution/

solution of Cs_2SO_4 (Anderman and Co. Ltd., London) in distilled water (ρ = 1.930gm/cm^{-3}) was added to give the required initial buoyant density as judged from the refractive index. In general, an increase in R^F of 0.05 led to an increase in initial buoyant density of 0.012gm/cm^{-3} and was achieved by increasing the amount of saturated Cs_2SO_4 added per ml of gradient by 0.013ml. The final DNA concentration was 50ug/ml solution.

Preparative centrifugation was carried out in volumes of 20ml/tube in the 8x40 fixed angle Titanium rotor of the MSE 65 ultracentrifuge. The solution was centrifuged at 30K rpm for 96 hours at 20°C, a hole was pierced in each tube, and fractions collected and diluted with 0.5ml 0.1M Na_2SO_4. Absorbance at 260nm was determined, fractions containing the regions of satellite DNA pooled, and this DNA re-centrifuged in Cs_2SO_4 with no additional Ag^+ added. After the second centrifugation, the satellite DNA regions were extensively dialysed against 5M NaCl plus 0.01M Tris-HCl pH7.0, followed by dialysis against 0.1xSSC. The purity of various satellite fractions was ascertained by analytical centrifugation in CsCl. All satellite DNA preparations were eventually dialysed against 0.1xSSC.

8. a) Fixation of cells onto coverslips

Cells, derived from monolayers in culture, were fixed in 3:1 methanol: acetic acid as previously described and air-dried directly onto coverslips.

b) In situ hybridisation with cells on coverslips

Hybridisation was carried out essentially as described above with the coverslip being sealed to a glass microscope slide with Cow Gum solution. Post-hybridisation, the coverslip containing the cells was eased from the slide and the Gum totally removed with the aid of watchmaker's/

watchmaker's forceps. The coverslips were then treated with RNase and exhaustively washed in cold 2xSSC as described for the routine in situ hybridisation procedure.

c) Scintillation counting of cells on coverslips

Processed post-in situ hybridised cells on coverslips were finally rinsed in alcohol and air-dried. These coverslips were then directly immersed in 10ml Toluene-based Scintillation fluid and the radioactivity determined.

d) Thermal melting of RNA-DNA and DNA-DNA duplexes with cells on coverslips

This was carried out as described for the thermal melting of hybrids retained on millipore filters (pg.42): i.e. the use of T.C.A. to precipitate either ^{3}H-labelled RNA or ^{3}H-Thymidine-labelled DNA.

SECTION V

1. Tumour Induction

Adenovirus 2 tumours were induced by inoculating Adenovirus 2 transformed cells into newborn Hooded Lister rats with or without immunosupression. (See Materials). Inoculation was carried out by Dr. P. H. Gallimore, Dept. Cancer Studies, Birmingham. Adenovirus 12 tumours were induced in newborn Hooded Lister rats by injecting purified Adenovirus 12 S.C. (See Materials). These tumours were excisable by about 8 weeks post-inoculation.

2. Preparation of virus-infected cells

Hela and HEK cells were used as permissive systems for the replication of Adenovirus 5, 2, 12, or 7 serotypes.

a) Plaque formation on cells

In some cases original Adenovirus 5 preparations were tested for infectivity by plaque formation on a monolayer of Hela cells overlaid with agar and stained with neutral red. The method was that of Williams (1970) which incorporates $MgCl_2$ in the medium, having an enhancing effect on the production of plaques.

Virus suspensions (diluted in PBS) were absorbed to Hela monolayers and the cells incubated for 90 minutes at 37°C. The monolayers were then overlaid with 5ml of 0.6% Noble agar (Difco) in Eagle's medium containing 2% FCS and 25mM $MgCl_2$. After incubation at 37°C in 5% CO_2 for 5 days, an additional 2ml of agar overlay medium was added to the cultures, and on the 6th or 7th day after infection a further 2ml of overlay medium containing neutral red was added. Normal cells stained with the neutral red and the plaques appeared clear and large.

b) Either Hela or HEK cells were grown to nearly 2/3 confluence in

90mm/

90mm or 50mm plastic Petri dishes. Virus (diluted in PBS) was added to the cells at defined m.o.i. and after the culturing medium had been removed. Virus was absorbed at 37°C for 30 min. - 3 hours in different experiments. After the required time, the cells were washed briefly in PBS and the growth medium then added. Cells were cultured at 37°C in 5% CO_2 in air.

3. Preparation of nuclear RNA from cultured tumour cells

Shimada et al.(1972) separated nuclear and cytoplasmic fractions of Adenovirus 2 transformed cells using modifications of the methods of Borun et al.(1967) and Penman (1966). Also they extracted nuclear RNA by a combination of the methods of Penman (1966), Fujinaga and Green (1967) and Warner et al.(1966). The method of Shimada et al. (1972) was used to separate nuclear and cytoplasmic fractions of Adenovirus tumour cells; and a combination of the methods of Penman (1966); Kirby (1965) and Parish and Kirby (1966) were used to extract the nuclear RNA.

a) Separation of nuclear and cytoplasmic fractions

Cultured cells were washed in Dulbecco A, Trypsinised, centrifuged and resuspended in $1x10^{-2}$M Tris-HCl, $1x10^{-3}$M $MgCl_2$, $1x10^{-2}$M NaCl, pH7.4 (RSB buffer). (Penman, 1966). Nonidet-P-40 (NP-40) (Shell Chemical Co.) was added to a concentration of 0.2% and the mixture incubated at 0°C for 15 minutes. (Shimada et al., 1972). The solution was then centrifuged at 2,200 rpm for 10 min. in the cold (4°C). The resulting pellet was resuspended in RSB + NP-40 and incubated for a further 5 minutes at 4°C. After agitation the suspension was again centrifuged at the previous speed for the same time and the nuclei pelleted.

The/

Table 11.2

Cell	Nonidet P-40%	Whole cell: nucleus + cytoplasm (x. 10^2cpm/ 100ml culture)	Cytoplasm (x10^2cpm/ 100ml culture/	Radioactivity in cytoplasm (%)
Adenovirus 2 transformed rat embryo (Ad2/REB/10p/B1)	0.2	10,736	279	2.6
"	0.2	10,378	291	2.8
* "	0.2	7,385	285	3.9

*Shimada et al. (1972)

The separation of nuclei and cytoplasm was evaluated by a) phase contrast microscopy and b) labelling cells with ^{3}H-Thymidine and determining the radioactivity in separated fractions. For the second determination the procedure was as follows: Cells were labelled with 0.5uCi/ml ^{3}H-Thymidine (20Ci/mmole Amersham) in Eagle's MEM, supplemented. After 20 hours incubation at 37°C, 5% CO_2 in air, the cells were rinsed with Dulbecco A and nuclear and cytoplasmic fractions prepared by the method outlined above. After addition of 2.5% Perchloric acid, the total acid-insoluble radioactivity is cytoplasmic (Table II:2). Shimada et al. (1972), using 0.2% NP-40, found less than 4% of the total radioactivity (^{3}H-Thymidine) in the cytoplasmic fraction.

4. Extraction of Nuclear RNA

1ml of Kirby's solution was added to nuclei and the mixture gently homogenised in a small Teflon homogenizer. An equal volume of phenol-cresol was added and the mixture shaken at room temperature for 20-30 minutes. This mixture was then centrifuged at 10,000 rpm for 10 minutes and the aqueous phase was removed. The phenol and proteic phases were then re-extracted with 2.5 volumes of distilled water and shaken for another 20-30 minutes at room temperature. After centrifugation at 10,000 rpm for 10 minutes the aqueous phase was removed and added to the first aqueous phase. This was now extracted with 1/2 volume of phenol-cresol, the phases separated by centrifugation, and the water phase re-extracted with 1/2 volume of chloroform to remove any phenol. 1/10 volumes of 2M sodium acetate (pH5.0) was added and the RNA precipitated with 2.5 volumes of absolute alcohol at -20°C overnight. The resulting precipitate was then pelleted by centrifugation at 10,000 rpm for

20/

20 minutes, the pellet washed in 1:1 alcohol: ether, and air-dried. 7mM $MgCl_2$, 50mM TrispH7.5 was added and the solution incubated with 50ug/ml of re-purified DNase (see pg.38) for 30 minutes at 0°C. After alcohol precipitation, the precipitate was stored at -20°C.

The RNA spectra was determined in an SP800 spectrophotometer and pure RNA gave an $E_{260}/E_{280} = 2$.

SECTION VI

1. Labelling of RNA in cultured cells

Adenovirus transformed cells or normal cells were grown in Eagle's MEM supplemented with 10% FCS and also containing 0.1% $NaHCO_3$, and antibiotic. The cells, when $\frac{1}{2}$ - $\frac{2}{3}$ confluent, were labelled with 10uCi/ml ^{3}H-Uridine (25Ci/m mole, Amersham) for 8 hours at 37°C. At the end of this labelling period the radioactive medium was removed and the cells washed in Dulbecco A.

2. Preparation of cytoplasmic labelled RNA from cultured cells

Nuclear and cytoplasmic fractions were prepared as previously described. To the RSB + NP-40 supernatant an equal volume of Kirby's solution was added. Phenol-chloroform-cresol was added to the mixture in a 1:1 ratio, and the RNA extracted by shaking at room temperature for 20 minutes. After centrifugation, the RNA was further extracted by the same procedure as has been already described for the preparation of nuclear RNA; and the final RNA solution precipitated with the addition of 1/10 volume 2M sodium acetate (pH5.0) and 2.5 volumes of absolute alcohol. The RNA was stored at -20°C overnight. All the preparative steps were carried out at 0-4°C.

3. Cellulose-oligo (dT) columns (Aviv and Leder, 1972)

1gm of cellulose-oligo (dT) binds 34 OD of poly A. 30mg were used to pack a sterile pasteur pipette. The column was washed with 10ml distilled water, 2ml of 0.1NaOH, 10ml of distilled water and finally 10ml of the binding buffer. (400mMNaCl, 1mM EDTA, 10mM Tris - 0.1% SLS pH7.6.) The RNA sample was suspended in the same binding buffer and loaded on to the surface of the column. The RNA molecules lacking poly A sequences were collected in fractions of binding buffer; the poly A-containing RNA molecules were eluted in low salt eluting buffer (1mM EDTA/

(1m M EDTA, 10mM Tris- 0.1% SLS pH7.6) and fractions collected. The radioactivity in the fractions of binding buffer and eluting buffer was monitored by counting fraction aliquots in liquid sinctillation fluid. (10ml Aquasol; NEN Chemicals, Germany). The RNA in the eluting was made 0.4M NaCl and the solution passed again through the column. Binding buffer and elution buffer fractions were again taken and the poly A-containing mRNA precipitated at -20°C.

4. Renaturation of ^{3}H-labelled Adenovirus 2 DNA

^{3}H-Thymidine labelled Adenovirus 2 DNA was prepared and purified as described previously.

The DNA was denatured by boiling at 100°C in 0.1xSSC for 30 minutes. Control samples were taken. The salt concentration was adjusted to 3xSSC and the renaturation carried out at 65°C. At various times throughout the reaction, samples were withdrawn and diluted into a large volume of cold SI nuclease buffer (0.03M sodium acetate pH4.5, $3x10^{-5}$ $ZnSO_4$, 0.01M NaCl). The diluted samples were then divided into equal portions and one portion treated with S1 nuclease enzyme (a gift from Dr. J. O. Bishop, this laboratory). S1 digests single strand DNA (Sutton, 1971). The treated and untreated samples were incubated at 50°C for 40 minutes, then placed on ice. 50ug of Bovine serum albumin per ml were added followed by 50% TCA to a final concentration of 10%. After 20 minutes on ice, these samples were filtered on GF/C filters, the filters washed with cold 5% TCA, dried and counted in Toluene-based scintillation fluid. The % of S1 nuclease-resistance for each sample was plotted against Log Cot.

5 Preparative CsCl centrifugation of labelled virus DNA

^{3}H-labelled Adenovirus 2 DNA was prepared as previously described.

A/

A small amount of virus DNA in 0.1xSSC, plus approximately 50ug human DNA and 15ug of M.Luteus marker DNA, was made up with 5.2gm CsCl (B.D.H., analytically pure) to an initial density of 1.700-1.720gm/cm^{-3}. The total volume was 4ml. Liquid paraffin was added to fill up the tube. The DNAs were centrifuged at 42K rpm for 40 hours at 25°C in an MSE 10x10 rotor. After centrifugation, tubes were pierced, fractions collected and diluted with 0.1xSSC. An equal volume of 1N NaOH was added and the DNA in each fraction denatured over a 15 minute period. DNA was then loaded onto membrane filters according to the method of Gillespie and Spiegelman (1965) and as already described. The radioactivity of each filter was counted in Toluene-based scintillation fluid, and the counts per fraction plotted.

6 In situ hybridisation of ^{3}H-Adenovirus DNA and Adenovirus-specific nucleic acids in eukaryote cells

The preparation of nuclear spreads and whole cells has already been described.

Adenovirus ^{3}H-DNA was denatured by heating at 100°C in 0.1xSSC for 30 minutes. The denatured DNA was rapidly chilled on ice, made up to 2xSSC, and added to cytological preparations at a concentration of usually 10^{-3}ug/5ul. Hybridisation was carried out for 10 hours at 65°C and the reaction terminated by chilling in ice-cold 2xSSC. Preparations were treated with S1 nuclease in 0.03M sodium acetate pH4.5, $3x10^{-5}$M $ZnSO_4$, 0.01M NaCl. After 30 minutes enzyme treatment at room temperature the slides were then washed in the S1 nuclease buffer followed by exhaustive washing in cold (4°C) 2xSSC. Dehydrated slides were coated with Ilford K2 emulsion and exposed as previously described. In some experiments, cells were RNased (20ug/ml; 2xSSC) before ^{3}H-DNA was added.

7. Preparation of ^{3}H-Poly (U)

The method is described in Jones, Bishop and Brito-da-Cunha (1973); and Bishop et al.(1974).

0.25mCi ^{3}H-UDP (13.3 Ci/m mole, Amersham) was lyophilised in a 10ml conical tube. 5ul of 0.5M Tris pH8.5 at 37°C, 0.1M KCl, 0.06M $MgCl_2$ were added and the tube shaken vigorously. 30ul of aqueous 1% UDP (Sigma) was added followed by 15ul polynucleotide phosphorylase (Miles-Seravac, Code No: 31/620.) Incorporation was carried out at 37°C and the reaction monitored by TCA precipitation of aliquots. When the incorporation reached a plateau, the reaction was terminated by adding 1.0ml of 0.2M sodium acetate pH5.0, 0.05mls 10% SLS and 0.5ml water-saturated phenol. The mixture was shaken for 2 minutes and 0.5ml of chloroform added. After a further shaking for 2 minutes the mixture was centrifuged, the aqueous phase removed, and the phenol phase re-extracted with 1ml of distilled water. The pooled aqueous phases were layered onto a Sephadex-G50 column previously equilibrated with 0.1xSSC. The effluent was monitored by scintillation counting and the peak fractions of ^{3}H-poly (U) pooled and lyophilised. The ^{3}H-poly (U) was dissolved in a solution which was finally 2-5xSSC. The specific activity of 3H-poly (U) was 2.5 x 10^{5}cpm/ug.

8. Hybridisation of ^{3}H-Poly (U) and Poly($_{r}$A) in Poly (U) Excess

50ng of cold poly ($_{r}$A) (Sigma), in distilled water, was made up and hybridised to ^{3}H-poly (U) in 2xSSC at 37°C. During the reaction, samples were taken and diluted 20 times in ice-cold 2xSSC. Two portions were removed and to one portion RNase (20ug/ml) was added. After 20 minutes in an icebath, bovine serum albumin carrier (250ug) and TCA (10%) were added. Samples were collected on GF/C (Whatman) filters, dried, and counted with 15ml of toluene-based scintillation fluid/.

fluid. The % RNase-resistance of each sample was calculated by comparing the RNased samples with the non-RNase treated ones.

9. In situ hybridisation of poly (U) and poly A-mRNA

Cytological preparations were denatured with 0.2N HCl as previously described, and prepared for in situ hybridisation. Approximately $1x10^4$cpm of ^{3}H-poly (U) were added in a 5 ul volume of 2xSSC. The reaction was carried out for 2-3 hours at 50°C, and terminated by washing the preparations in ice-cold 2xSSC. RNasing (20ug/ml) was carried out at 4°C for 20 minutes and the preparations washed exhaustively in 2xSSC at 4°C. After the last cold 2xSSC wash, the slides were treated as previously described.

CHAPTER III

ATTEMPTS TO DETECT ADENOVIRUS DNA IN ADENOVIRUS TRANSFORMED AND TUMOUR CELLS: PHYSICAL METHODS AND THE USE OF VIRUS COMPLEMENTARY RNA

SECTION 1

BASIC BIOPHYSICAL STUDIES

The techniques of buoyant density centrifugation in CsCl and thermal denaturation can be particularly useful in discriminating classes of DNA within the eukaryote genome. CsCl buoyant density centrifugation, for example, is capable of resolving certain satellite DNAs (Walker, 1970; Flamm, 1972); and thermal gradient denaturation of some DNAs result in the resolution of ribosomal genes (Birnstiel et al, 1970). DNA-DNA reassociation as well can discriminate classes of DNA which in this case are defined on the basis of their base-sequence repetitition frequency (Britten and Kohne, 1968; Walker, 1970). The subject of this section is whether virus DNA can be resolved from the rest of the genomal DNA in certain Adenovirus transformed or tumour cells by these techniques.

Results and Conclusions

a) Buoyant density determinations in neutral CsCl.

DNAs were spun in the analytical ultracentrifuge and their buoyant densities determined (Table III:1).

There is no difference in the values for normal DNA, both Adenovirus 2 and 7 transformed cell DNA, and DNA from tumours induced by Adenovirus 2 transformed cells or Adenovirus 12. These DNAs all possess buoyant density values of I.699 (1.700) gm/cm^{-3}.

Table III:1

DNA	* Buoyant density (gm/cm^{-3}) in neutral CsCl	(G+C)%	(G+C%) OTHER STUDIES
Mouse	1.699/1.690	39.0 main/34.2 satellite	Flamm et al. (1967)
Rat	1.700	39.0-40.0	40.0 (Steele, 1968)
E. coli	1.712	51	
M. luteus	1.731	72	
Xenopus ribosomal (oocyte)	1.729	66-68	70 (Birnstiel et al., 1970)
Human	1.699	39.9	
Adenovirus 2	1.716	58	57-59)
Adenovirus 7	1.711	50	49-52) (Green, 1970)
Adenovirus 12	1.708	48	48-49)
Ad2/REB/10p/B1	1.700	40	
Ad2/REB/50p/B1	1.700	39.0-40.0	
Ad2/T4	1.699	39.9	
Ad2/T5	1.700	40.0	
Ad2/T6	1.699	39.9	
Ad7/1	1.700	40	
Ad12/T1	1.700	39.0-40.0	

* Analytical ultracentrifuge

The three Adenovirus DNAs-2, 7 and 12 - possess unique buoyant density values, and the calculated GC contents are in agreement with previously determined values for these particular virus DNAs (Green, 1970).

Mouse DNA, as expected, runs as two detectable peaks in the gradient: 1.699gm/cm^{-3} (main band) and 1.690gm/cm^{-3} (AT-rich satellite) (Kit, 1961; also this Thesis, pg. 102).

The molecular weights of the DNAs are all in the range of 10^7 daltons, and decreasing this value by a 1000 fold failed to resolve any differences between the virus transformed, the tumour, and normal rat DNA (data not shown).

b) Thermal dissociation of DNAs.

Several DNAs were melted in 1 x SSC pH 7.5, or ImM EDTA. The latter solution was preferable since the melting range of the DNAs was lower than with the SSC solution and consequently reduced boiling of the DNA solution in the spectrophotometer cuvettes. The use of this EDTA solution has already been noted (Spiers, unpublished) and it gave reproducible results with a variety of DNAs (Figures III:1 and III:2). Bacterial DNAs of known GC contents and Tms* served as standards (see Figure III:2).

There is no difference in the Tms of normal rat DNA, transformed DNA or tumour DNA (Table III:2); and Adenovirus DNA isolated from serotypes 2, 7 and 12 each possess Tms which are consistent with their GC contents, and with values obtained by other workers (Green and Pina, 1964; Green, 1970).

---

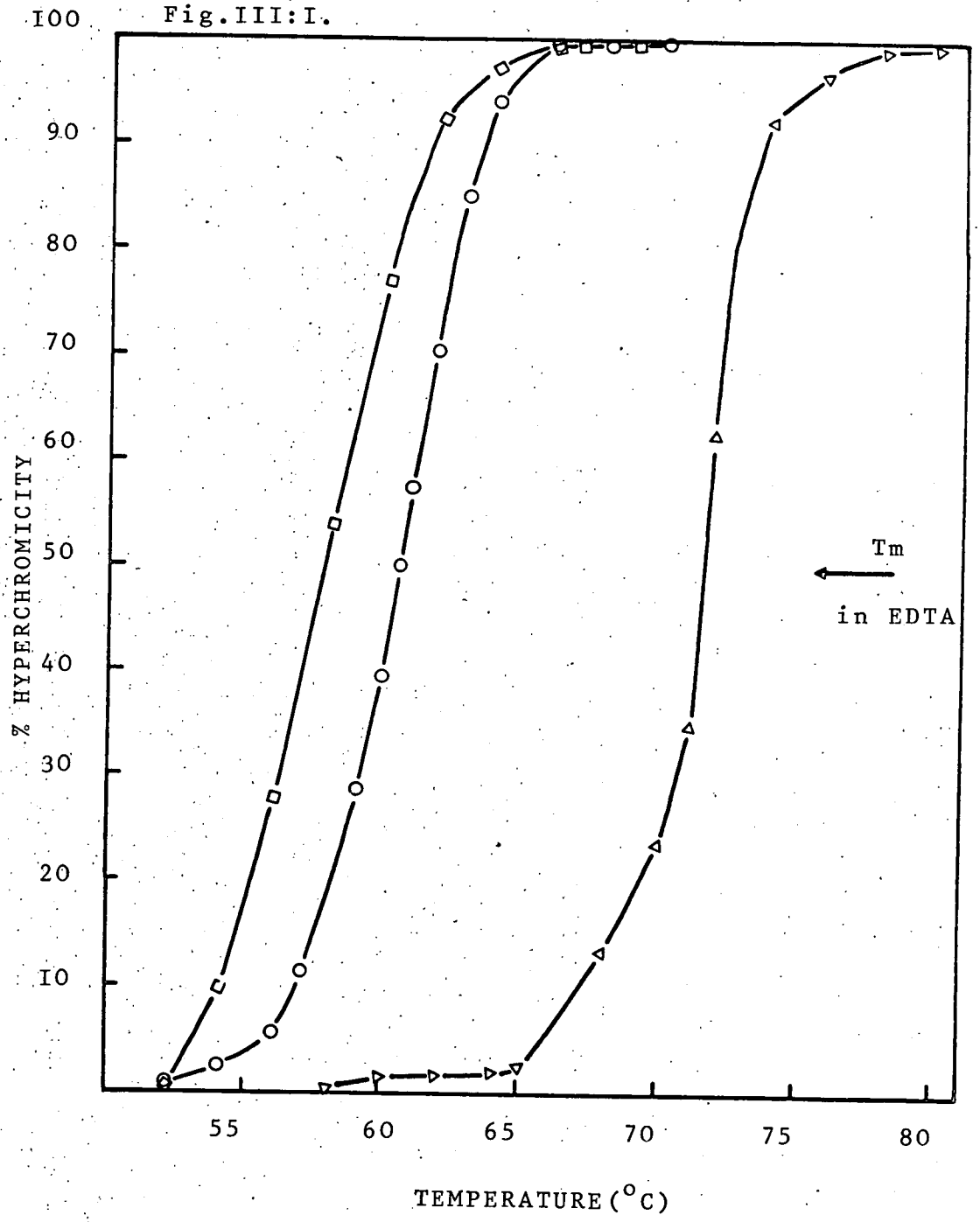
* Tm = mid-point of the DNA melting transition (^{o}C)

Table III:2

DNA	Tm DNA (1mM EDTA)	Tm(1xSSC)	Tm(1xSSC) Other Studies
Micrococcus luteus	72	99	99(Spiers,1973)
E. coli	61.5	90	
mouse	59.5	85.5	
rat	58.1	85.5	
human	58.1	85.5	
chimpanzee (pan troglodytes)	58.5	85.5	86(Prosser,1974)
Adeno-2 transformed (Ad2/REB/10p/B1)	58.1	85.5	
" " (Ad2/REB/50p/B1)	58.1	85.5	
Adeno-7 transformed (Ad7/1)	58.1	85.5	
Adeno-2 Tumour (Ad2/T4)	58.1	85.5	
Adeno-2 Tumour (Ad2/T5)	58.1	85.5	
Adeno-2 Tumour (Ad2/T6)	58.1	85.5	
Adeno-12 Tumour (Ad12/T1)	58.1	85.5	
Adenovirus 12	61	89	88.8(Green and)
Adenovirus 2	66	92.5	92.4(Pina, 1964)
Adenovirus 7	62	90	

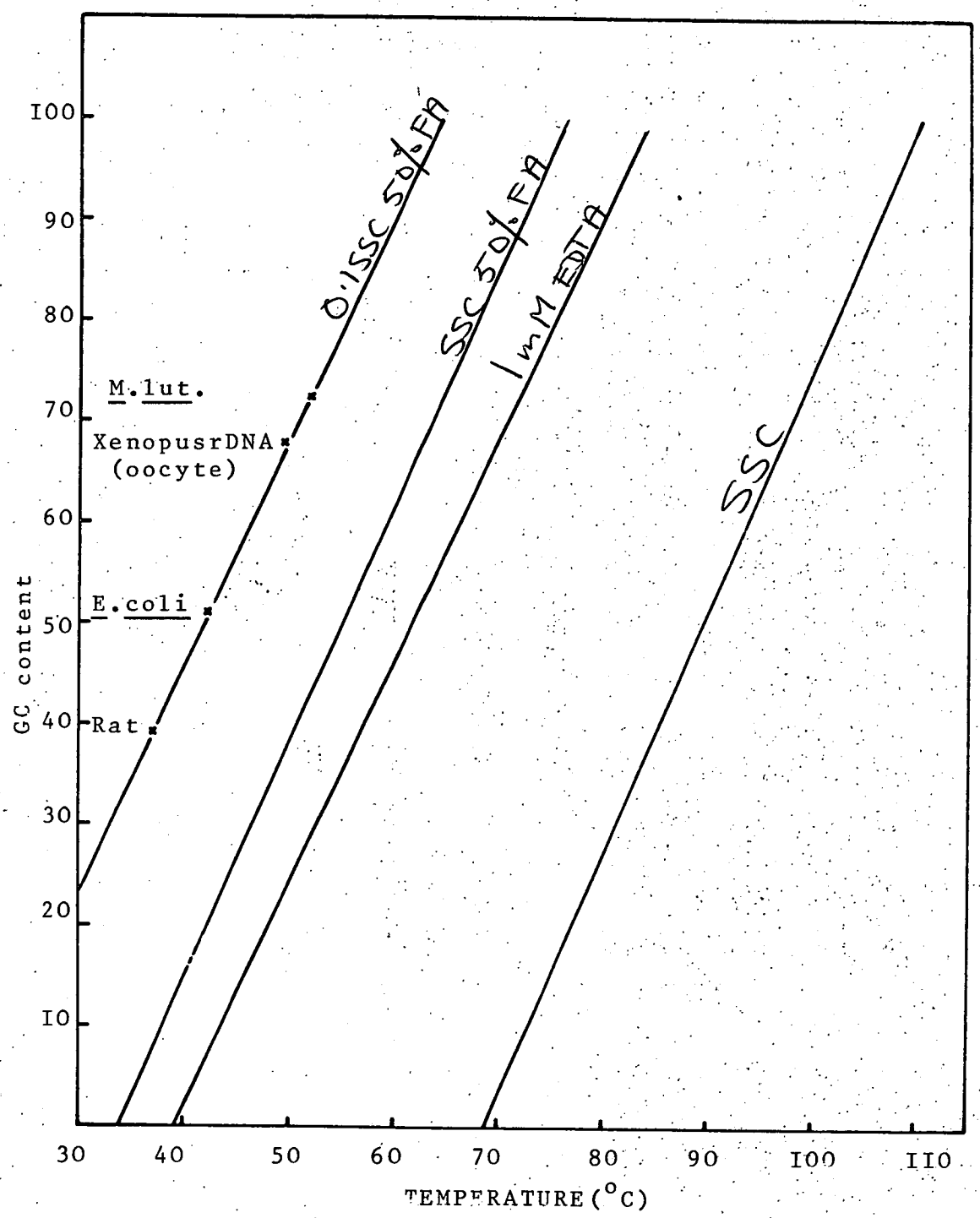
Figure III:I. Melts of DNA in a ImM EDTA solution.Each DNA was dialysed against ImM EDTA and samples concentrated to 25ug/ml.

Fig.III:I.


100
90
80
70
60
50
40
30
20
10
% HYPERCHROMICITY
55
60
65
70
75
80
TEMPERATURE (°C)
Tm
in EDTA
Rat DNA
M.luteus DNA
E.coli DNA

FigureIII:2. Tms for various DNAs in different solutions.DNA standards were M.luteus,Xenopus laevisrDNA(oocyte), E.coli,and rat.

Fig.III:2.


100
90
80
70
60
50
40
30
20
10
GC content
M.lut.
XenopusrDNA
(oocyte)
E.coli
Rat
0.15SSC 50% FA
SSC 50% FA
1mM EDTA
SSC
30
40
50
60
70
80
90
100
110
TEMPERATURE (°C)

c) Reassociation kinetics

DNA was denatured and reassociated according to procedures outlined in Materials and Methods. The DNA was sonicated to a Molecular Weight of 1.5×10^5 daltons and reassociation carried out at 70°C in 2 x SSC. The data are presented as conventional log Cot curves, the $Cot\frac{1}{2}$ being a measure of the complexity of the individual DNAs (Britten and Kohne, 1968).

Normal rat DNA reassociates over a wide range of log Cot values, a finding which is consistent with other studies on rat DNA (Melli et al., 1971; Campo, 1973); and DNAs from tumour or transformed cells exhibit the same reassociation transitions (Figures III:3 and III:4). There is no increase or decrease in the frequency of highly repetitive, intermediate or unique DNA sequence - the three main classes of DNA definable by this method - between normal rat, transformed or tumour DNA. In fact, all these DNAs show two main transitions; one at a $Cot\frac{1}{2}$ of around 10 and another around a $Cot\frac{1}{2}$ of 1000. These two transitions represent a proportion of the genome comprised of a heterogeneous population of highly reiterated sequences and a larger proportion composed, for the most part, of unique sequences only.

The three techniques used here have therefore failed to resolve any differences in the DNA from normal cells and transformed or tumour cells. These particular techniques, however, have limited resolving power (see e.g. Walker, 1970; Flamm, 1972) and therefore the failure to resolve any differences between normal DNA and transformed or tumour DNA could reflect these limitations. Experiments were therefore designed which utilised the base-sequence specificity of the Adenovirus DNAs. Radioactive complementary RNAs (cRNAs) to the Adenovirus

Figure III:3. Reassociation of DNA in 2xSSC, at 70°C over several Cot values. DNA was at a concentration of 20mg/ml. Reassociation was calculated according to the formula:

$$\frac{E_{26o(9o)} - E_{26o(5o)} \times 100}{E_{26o(9o)} - E_{26o(9o)} \times 100} \Big/ 38$$

as described in Materials and Methods, pg. 33

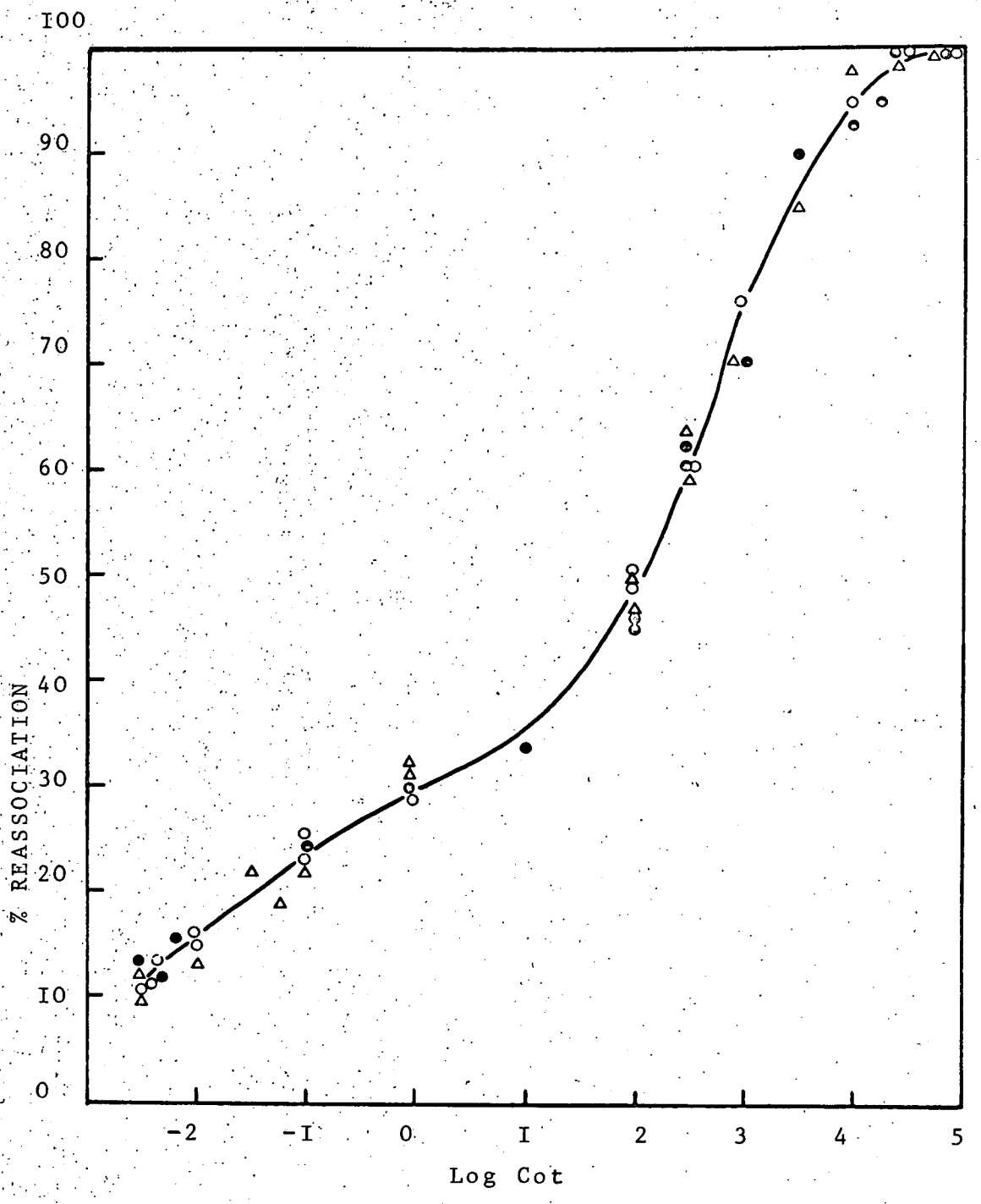

100
90
80
70
60
50
40
30
20
10
0
% REASSOCIATION
-2
-1
0
1
2
3
4
5
Log Cot
Normal rat DNA
Ad2/REB/IOp/BI
Ad2/T5

Fig.III:3.

Figure III:4. Reassociation of DNA in 2xSSC at 70°C.20mg/ml DNA:% reassociation was calculated according to the formula as presented in Figure III:3.Figures III:3 and III:4 show that reassociation of normal rat DNA parallels reassociation of transformed cell or tumour cell DNA.

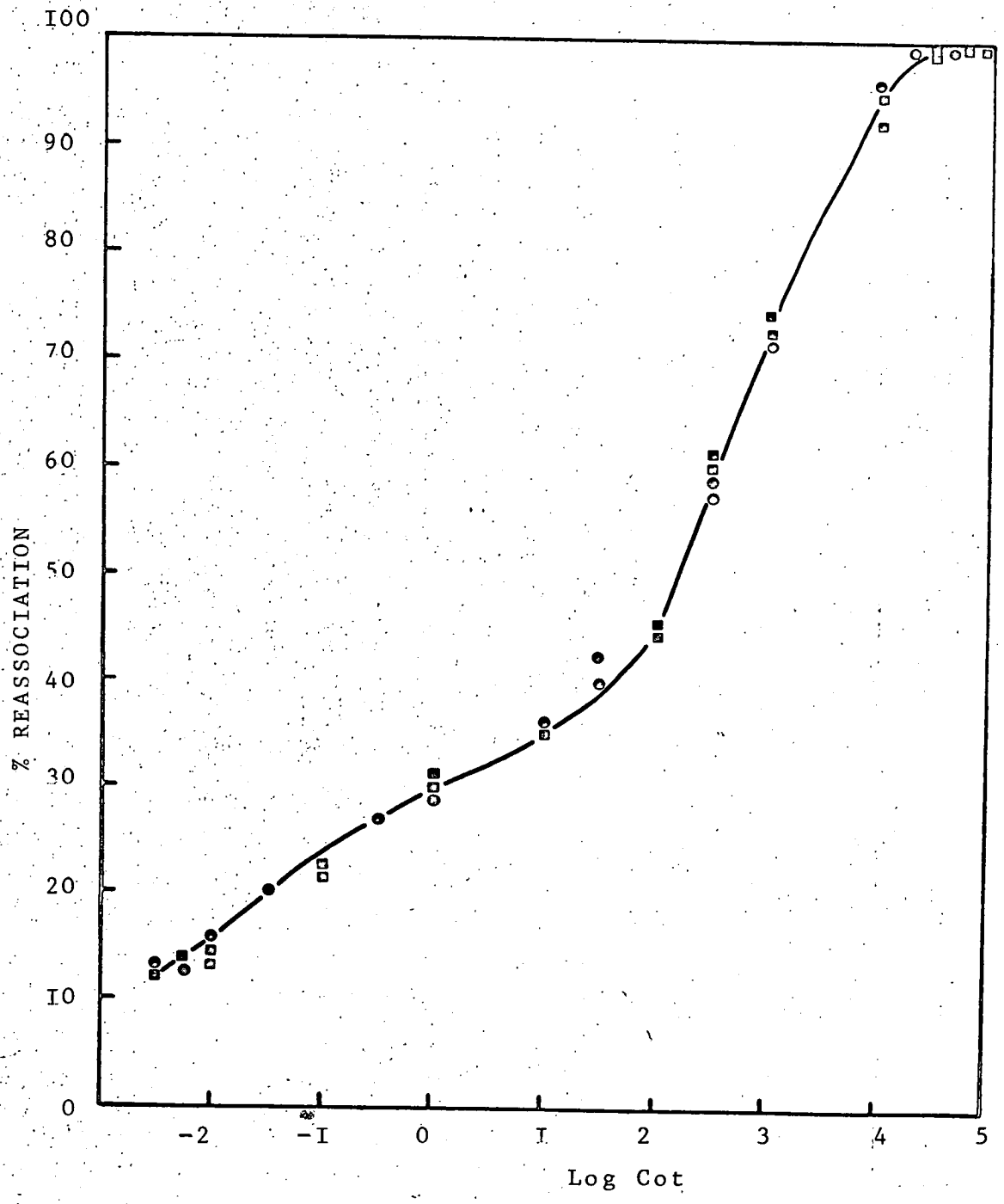

100
90
80
70
60
50
40
30
20
10
0
% REASSOCIATION
-2
-1
0
1
2
3
4
5
Log Cot
Ad7/I
AdI2/T1

Fig.III:4.

DNAs were synthesised and used to try and detect virus-specific DNA sequences in transformed or tumour DNA. The preparation, characterisation and use of these cRNAs in detecting such DNA sequences are described in the following sections.

SECTION II

BASIC PROPERTIES OF THE TRANSCRIPTION OF ADENOVIRUS DNA BY THE E.COLI DNA-DEPENDENT RNA POLYMERASE

This section deals with some of the basic properties of the transcription of Adenovirus DNA by the E. coli DNA-dependent RNA polymerase (E. coli RNA polymerase). While this polymerase's action on certain DNAs is well documented (see e.g. Richardson, 1969), little is known about its mode of action on Adenovirus DNA.

In general, the amount and rate of synthesis of RNA produced in vitro is dependent on a variety of factors which are either related to the nature of the template DNA or to the mode of action of the enzyme itself. Thus transcription is dependent on the frequency and availability of initiation and termination sites on the DNA for example; and is also dependent on the rate of chain growth or polymerisation. Each of these processes in turn have their own specific rates which can be additionally influenced by factors such as the substrate concentration, the ionic strength, the availability of polymerase molecules and the nature of the DNA. Reflecting this, RNA synthesis can be enhanced in the presence of high ionic strength for example (So et al., 1967; Bremer, 1970; Maitra and Barash, 1969); Salmon Sperm DNA is transcribed more efficiently than Calf Thymus DNA (Chamberlain and Berg, 1962); and an increased superhelicity of the closed circular DNA of phage λ leads to increased amounts of RNA synthesis (Botchan et al., 1973).

One of the points about the existence of these various influences is the fact that until they have been recognised and characterised it is unclear whether specific transcription reactions are being carried

out under conditions which maximise incorporation. This is an important point since it is frequently desirable to define conditions of RNA synthesis: for example, very poor transcription may be due to unfavourable ionic strength or alternatively to infrequent initiation sites on the DNA and failure to be aware of the first effect might erroneously lead to explanation of poor transcription on the basis of infrequent initiation sites only. Moreover, apart from this general consideration, it is clearly desirable to have transcription conditions which favour maximal amounts of RNA synthesis since the production of highly radioactive cRNAs for use in nucleic acid hybridisation experiments is an expensive process.

This section deals therefore with some aspects of the transcription of Adenovirus cRNAs principally by E. coli RNA polymerase. The results are particularly important in that they show that Adenovirus cRNA synthesis has similar reaction dependencies as those described for a variety of other DNA-E. coli RNA polymerase interactions. This means, in effect, that subsequent use of Adenovirus cRNAs to detect regions of DNA homology (see later) does not have to rely on peculiar or unique conditions of cRNA synthesis.

Results and Discussion

The amount of RNA synthesised can be measured by its precipitation with trichloroacetic acid (TCA), the filtered radioactivity being counted in a Toluene-base scintillation fluid. The rate of RNA synthesis can be measured by the size of RNA transcripts produced in a short incubation time. This size, which is a measure of the number of nucleotides incorporated, can be determined from the RNA's sedimentation in sucrose density gradients.

1. Like all RNA polymerases, the reaction with E. coli polymerase requires a cation, the four ribonucleoside triphosphates, and a DNA primer (Fox and Weiss, 1964; Richardson, 1969). The basic mix (see Figure III:5 legend) also includes Spermidine (1.6mM) which helps to stabilise the enzyme and stimulate the rate and extent of RNA synthesis (Fox and Weiss, 1964; So et al., 1967). Enzyme was added at saturating concentrations which, under optimum conditions, would theoretically be capable of catalysing the incorporation of 2.5n moles ^{14}C-ATP into acid-insoluble material in the presence of Calf Thymus DNA in 10 minutes. The basic mix also contains the buffers Tris and K_2HPO_4.

2. The DNA and cation dependencies essential for nucleotide incorporation into RNA chains are shown in Figure III:5. When Mg^{++} and Mn^{++}, or DNA is omitted from the incubation mix, there is little TCA precipitable material in 30 mins. of incubation at 37°C (Figure III:5a). When Mg^{++} or Mn^{++} and DNA are added, however, there is transcription (Figure III:5b) which is more extensive when both Mg^{++} and Mn^{++} are present as well as DNA (Figure III:5a). Chamberlin and Berg (1962), in studying the action of E. coli RNA polymerase on a variety of DNAs, also found that the effect of Mg^{++} and Mn^{++} is accumulative in this way. Under normal conditions of transcription the reaction essentially plateaus at approximately 30 mins. incubation at 37°C. Similar in vitro kinetics, using this enzyme, have been reported for bacteriophage DNA (Richardson, 1969); a variety of eukaryotic DNAs (see Jones, 1973 for example); and certain DNA virus DNAs (see Pettersson et al., 1974 for example).

Figure III:5.Synthesis of Adenovirus 2cRNA. The basic incubation mix contains:I-2ug DNA(dialysed against 0.00IM NaCl),0.IM Tris pH7.5,Spermidine(I.6mM),0.09-0.IM KCL,2mM K_2HPO_4,5 nmoles each of ATP, UTP,CTP,GTP(either all labelled with tritium:I5-20Ci/mmole,or one labelled only:usually UTP),and 2.5 units enzyme. In experiments with all four triphosphates labelled the results were the same although the radioactivity incorporated was higher. At the termination of the reaction samples were TCA precipitated and the radioactivity determined by counting in Toluene-based scintillation fluid.(Toluene-PPO,POPOP). III:5a) o-o basic mix (all four nucleotides labelled) plus 0.0005M $MnCl_2$, 0.004M $MgCl_2$;x-x as for o-o but DNA denatured;+ − + basic mix only; ⊙—⊙ basic mix minus DNA but containing 0.0005M $MnCl_2$ and 0.004M $MgCl_2$.
III:5b) o-o basic mix(all four nucleotides labelled) plus 0.004M $MgCl_2$;+ −+basic mix (all four nucleotides labelled) plus 0.0005M $MnCl_2$.Same sample volumes taken as for Figure III:5a.,and the RNA TCA precipitated.

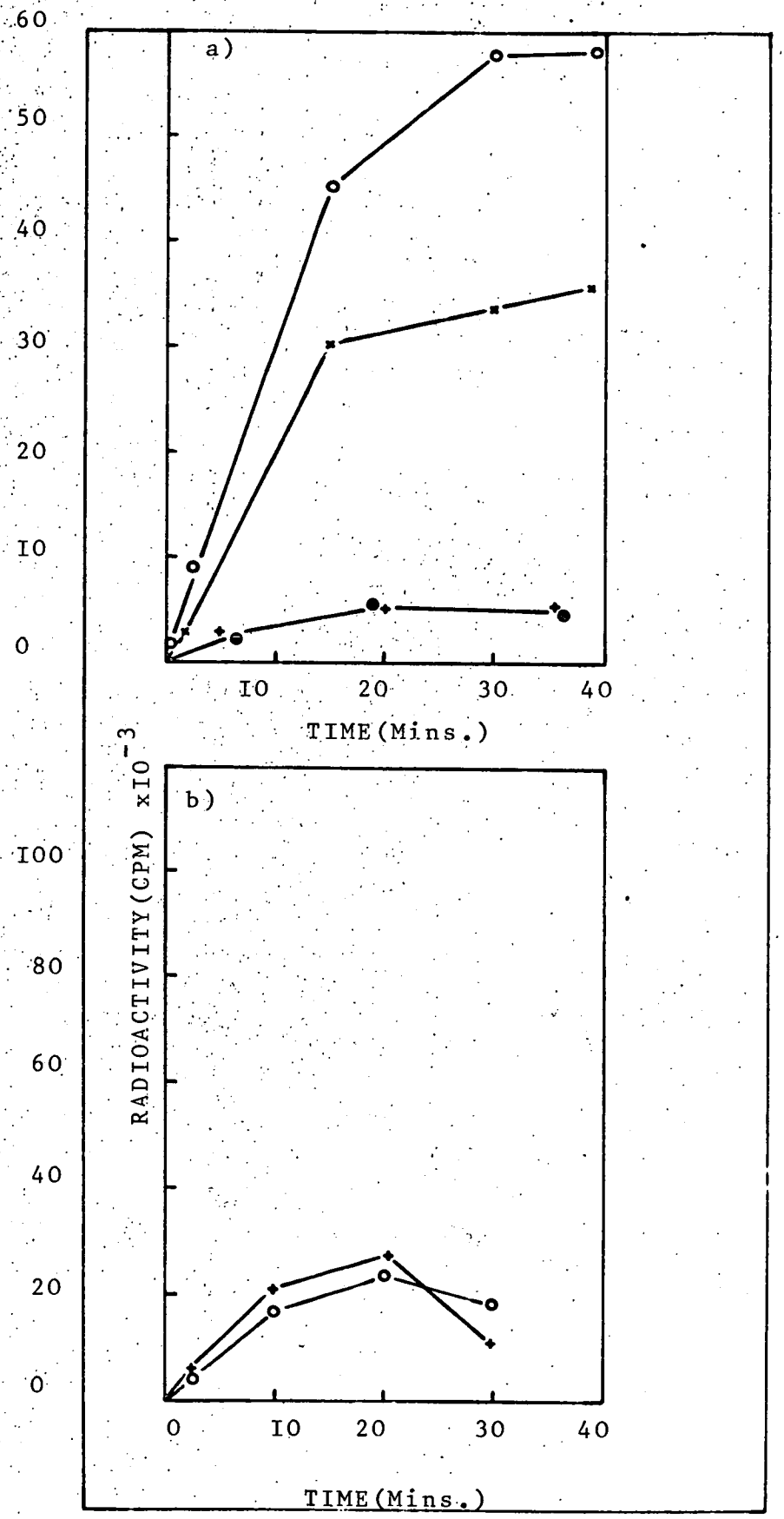
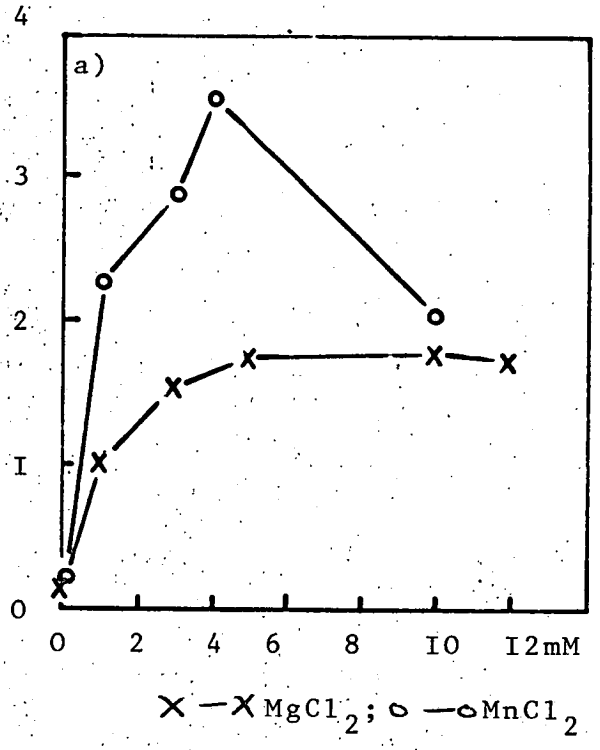

a)
60
50
40
30
20
10
0
10
20
30
40
TIME(Mins.)
b)
RADIOACTIVITY(CPM) x10^-3
100
80
60
40
20
0
0
10
20
30
40
TIME(Mins.)

Fig.III:5.

Denatured Adenovirus DNA (heating at 100°C in 0.001M NaCl for over 30 mins. prior to transcription) shows a marked reduction in RNA synthesis (Figure III:5a) which although not as much as for denatured T6 DNA (Chamberlin and Berg, 1962) or the transcription of denatured rDNA by the M. luteus DNA-dependent RNA polymerase (Hecht, 1973), is consistent with the lower levels of RNA synthesis observed when, in general, any template DNA is denatured (see Richardson, 1969 for example) .

3. At different concentrations of Mg^{++} or Mn^{++} transcription of Adenovirus 2 DNA proceeds at different rates, the cation optimums being 4mM Mn^{++} or above 4mM Mg^{++} (Figure III:6a). These optimums are in agreement with those found for the action of E. coli polymerase on Salmon Sperm DNA (Chamberlin and Berg, 1962), Calf Thymus DNA (Furth et al., 1962) and the rDNA of Xenopus laevis (Reeder and Brown, 1970). In addition, RNA synthesis does not seem to be impaired at relatively high concentrations of 10-12mM Mg^{++} (Figure III:6a) and this finding is consistent with other studies where concentrations as high as this have been used without inhibition of RNA synthesis; for example with T4 DNA (Bremer, 1970). The addition of Mn^{++} rather than Mg^{++} appears to lead to greater stimulation (also Figure III:6a). The cation dependence for the in vitro transcription of some other DNAs also exhibits this predilection for Mn^{++} (Maitra et al., 1967). (When both Mg^{++} and Mn^{++} were present in the incubation mix the same incorporation of RNA as obtained for optimal Mn^{++} alone was obtained with low concentrations of Mn^{++} (2mM) together with Mg^{++} concentrations of around 4-8mM (data not shown). This finding is also in agreement with those obtained by other workers (e.g. Chamberlin and Berg, 1962).)

Figure III:6. Titration of Adenovirus 2 cRNA synthesis with either $MgCl_2$ or $MnCl_2$ and also KCl.a) basic incubation mix (UTP labelled only) plus $MgCl_2$ (x-x) or $MnCl_2$ (o-o).KCl at O.IM. RNA TCA precipitated after IOmins. incubation at 37°C.b)Basic mix(UTP labelled only) plus $MgCl_2$ (4mM) and increasing amounts of KCl. Reaction terminated after IOmins. incubation at 37°C and RNA TCA precipitated(IO%).


a)
4
3
2
I
0
0 2 4 6 8 IO I2mM
× —× $MgCl_2$; o —o $MnCl_2$

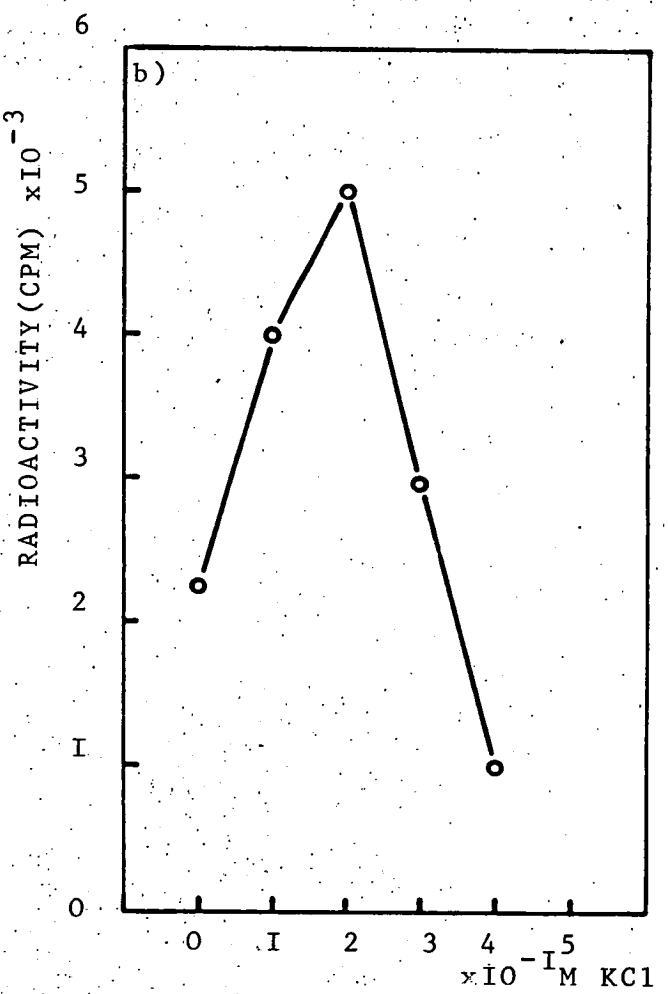

b)
RADIOACTIVITY(CPM) xIO^{-3}
6
5
4
3
2
I
0
0 I 2 3 4 5
xIO^{-I}M KC1

Fig.III:6.

4. The amount of RNA chains synthesised *in vitro* with the *E. coli* RNA polymerase is dependent on the salt concentration (Bremer, 1970) so that at higher ionic strength (0.2M KCl) more RNA is produced than at lower ionic strength (e.g. 0.05M KCl).

This is mainly the result of re-initiation of RNA chains (Richardson, 1969); an increased growth rate (Bremer, 1970); and a decline in end-product inhibition (So *et al.*, 1967) at elevated ionic strength. In order to see whether high ionic strength enhanced Adenovirus cRNA synthesis, Mg^{++} - containing incubation mixes were titrated with increasing amounts of KCl. Figure III:6b shows that the amount of RNA synthesised at 0.2M KCl is greater than at other ionic strengths while concentrations higher than 0.25M are inhibitory. This latter concentration of KCl is also inhibitory for the synthesis of T4 DNA (So *et al.*, 1967), amongst others.

At 0.2M KCl with Mn^{++} present, however, the reaction is slightly inhibited and reducing the molarity of the KCl to around 0.1M when Mn^{++} was present as well as Mg^{++} gave the most optimum results (data not shown).

As mentioned, the actual growth of RNA chains is faster in high ionic strength. T4 cRNA synthesis, with *E. coli* RNA polymerase, for example is 2.5 nucleotides per second when incubated in low salt (0.3M KCl) but is 36 nucleotides per second in high salt (0.2M KCl) and with saturating concentrations of nucleoside triphosphates (Bremer, 1970). In high salt, 35 nucleotides per second have been reported for polyrAU formation (Geiduschek and Haselkorn, 1969) and 16-20 per second have been recorded for λ cRNA synthesis (Richardson, 1969). A slow rate of cRNA synthesis in high salt (0.15M) is

characteristic of ribosomal DNA however. Reeder and Brown (1970) for instance report 2-3 nucleotides per second for the rate of RNA synthesis with E. coli RNA polymerase on Xenopus rDNA.

Figure III:7 shows that the rate of Adenovirus cRNA (0.15M KCl) synthesis is faster than the rate of transcription of rDNA (0.15M). An approximate rate of Adenovirus cRNA synthesis can be estimated from the size of this virus cRNA produced in a short incubation time. Figure III:8a shows that after only 60 seconds incubation, 28S RNA transcripts exist which must contain about 4.5×10^3 nucleotides (1.6×10^6 daltons) and an approximate minimum transcription rate of 75 nucleotides per second can therefore be calculated for the synthesis of Adenovirus 2 cRNA. This rate is comparable with many other in vitro transcription rates using E. coli RNA polymerase.

5. The size of the Adenovirus cRNA synthesised by E. coli RNA polymerase has been studied both here and by other workers. In the experiments described here there are several peaks of radioactivity along a sucrose gradient, the largest peak occurring around 28S (Figure III:8b). This is also true for Adenovirus 12 cRNA (Figure III:8c). Pettersson et al. (1974), using DMSO-sucrose gradients, have estimated the cRNA transcribed off Adenovirus 2 DNA, by the E. coli RNA polymerase, to be around 28S while Loni and Green (1973) using 3.2% polyacrylamide gels, have obtained several discrete Adenovirus 2 cRNA peaks which fall into a molecular weight range somewhat lower than that reported here or by Pettersson et al. (1974); their S values being 8S, 12S, 13S and 16S. Loni and Green (1973) also report that Adenovirus 7 and 12 cRNA both migrate in 3.2% polyacrylamide gels at 9 and 12S values which are again lower than those

Figure III:7. Synthesis of Adenovirus 2cRNA and Xenopus cRNA to rDNA. Incubation mix contains 0.I5M KCl, I.6mM Spermidine, 0.004M $MgCl_2$, 2mM K_2HPO_4, 5nmoles ATP, CTP, UTP, GTP (all labelled with tritium at I5-20cI/mmole), 2.5 units polymerase, I00mM Tris pH7.5; and x-xIug Adenovirus 2 DNA (dialysed against 0.00IM NaCl) or o-o Iug Xenopus rDNA (dialysed against 0.00IM NaCl). Aliquots were withdrawn at specific times of incubation at 37°C and the RNA TCA precipitated and its radioactivity determined by counting in Toluene-based scintillation fluid.

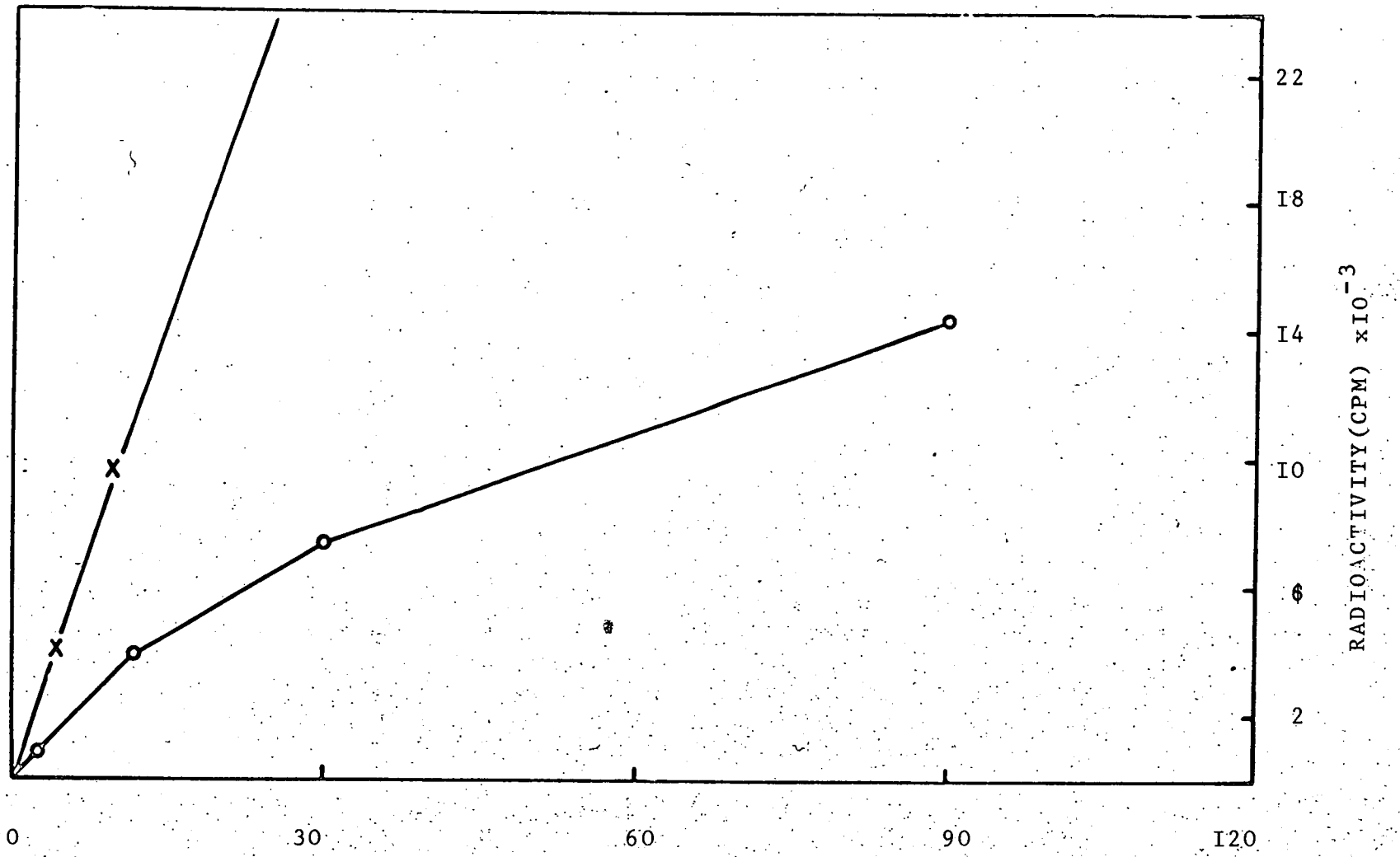

22
I8
I4
I0
6
2
RADIOACTIVITY(CPM) xIO^{-3}
0
30
60
90
I20
INCORPORATION TIME(Mins.)

Fig.III:7.

reported here. Lower estimates of 4S for the size of Adenovirus cRNAs (McDougall et al., 1975) may be due to lyophilisation (Figure III:8a) or to freezing at -70°C which can decrease the molecular size of RNA.

The Adenovirus genome is around 20-25 x 10^6 daltons (Green et al., 1967) and RNA transcripts approaching this size would be considerably greater than 28S in a sucrose gradient. That no very large molecular peaks in such gradients have been observed (Figure III:8a, 8b, 8c; see also Pettersson et al., 1974; Loni and Green, 1973) could be due to premature digestion of cRNA molecules by nuclease present, nicks in the template DNA, or preferential or interrupted transcription. Selective transcription of Adenovirus DNA is partially the subject of the following section of this Thesis, and it has also been observed by other workers (Pettersson et al., 1974; Green and Hodap, 1972; Dunn et al., 1973). Pettersson et al. (1974), in particular, have demonstrated that initiation of RNA synthesis by E. coli RNA polymerase appears to occur in at least five locations on Adenovirus 2 DNA as determined by electron microscopy of transcription complexes, some sites being more active than others. Two regions which correspond to 7.11% and 4.7% of the Adenovirus DNA duplex appear to be particularly active. Either region does not exceed 1.0 x 10^6 daltons in single strand molecular length and an RNA transcript from such a region would therefore not approach 28S in sucrose gradients. However RNA appears to be synthesised from other regions as well, one region amounting to 59.8% of the virus genome. That transcripts larger than equivalent to 28S fail to be detected in the sucrose gradient experiments described here and elsewhere (see Pettersson et al., 1974; Loni and Green, 1973)

Figure III:8. Size distribution of Adenovirus cRNA transcripts in sucrose density gradients. cRNA in 0.00IM EDTA,0.IM NaCl,DEP(0.I%),0.2 M sodium acetate was sedimented through a sucrose density gradient(5-40%) made up in the same buffer.The gradients were spun at 25,000 rpm for I8 hours at IO-I5°C in the 6xI5 rotor (MSE).Fraction aliquots were TCA precipitated and the radioactivity determined by counting in Toluene-based scintillation fluid.E .coli RNA was added to each gradient as a density marker(arrows).III:8a)△—△ Adenovirus 2cRNA synthesised in a short incubation time of 60seconds.The transcription mix contained IOOmM Tris pH7.5,5 n moles each of ATP,CTP, GTP,UTP(all labelled with tritium:I5-20 cI/mmole) ,2.5 units enzyme,2mM K_2HPO_4,I.6mM Spermidine, 0.I5 M KCl,0.004M $MgCl_2$ and I-2ug DNA(0.00IM NaCL). The reaction,at 37°C,was stopped after 60 seconds, RNA precipitated with alcohol and finally resuspended in gradient buffer;▣-▣ Adenovirus 2cRNA synthesised as in△—△but reaction stopped after 30mins. and cRNA lyophilised after ex--traction,before addition to gradient.III:8b) o-o Adenovirus 2 cRNA synthesised after 30mins. incubation in basic mix but with 4mM $MgCl_2$, 0.IM KCl ,0.0005M $MnCl_2$,5nmoles ATP,CTP,UTP, GTP(all tritium labelled) added.cRNA was alcohol precipitated after extraction, before addtion to gradient buffer;III;8c) Adenovirus I2 cRNA synthesised in same mix as III:8b.See text and Materials amd Methods for details.

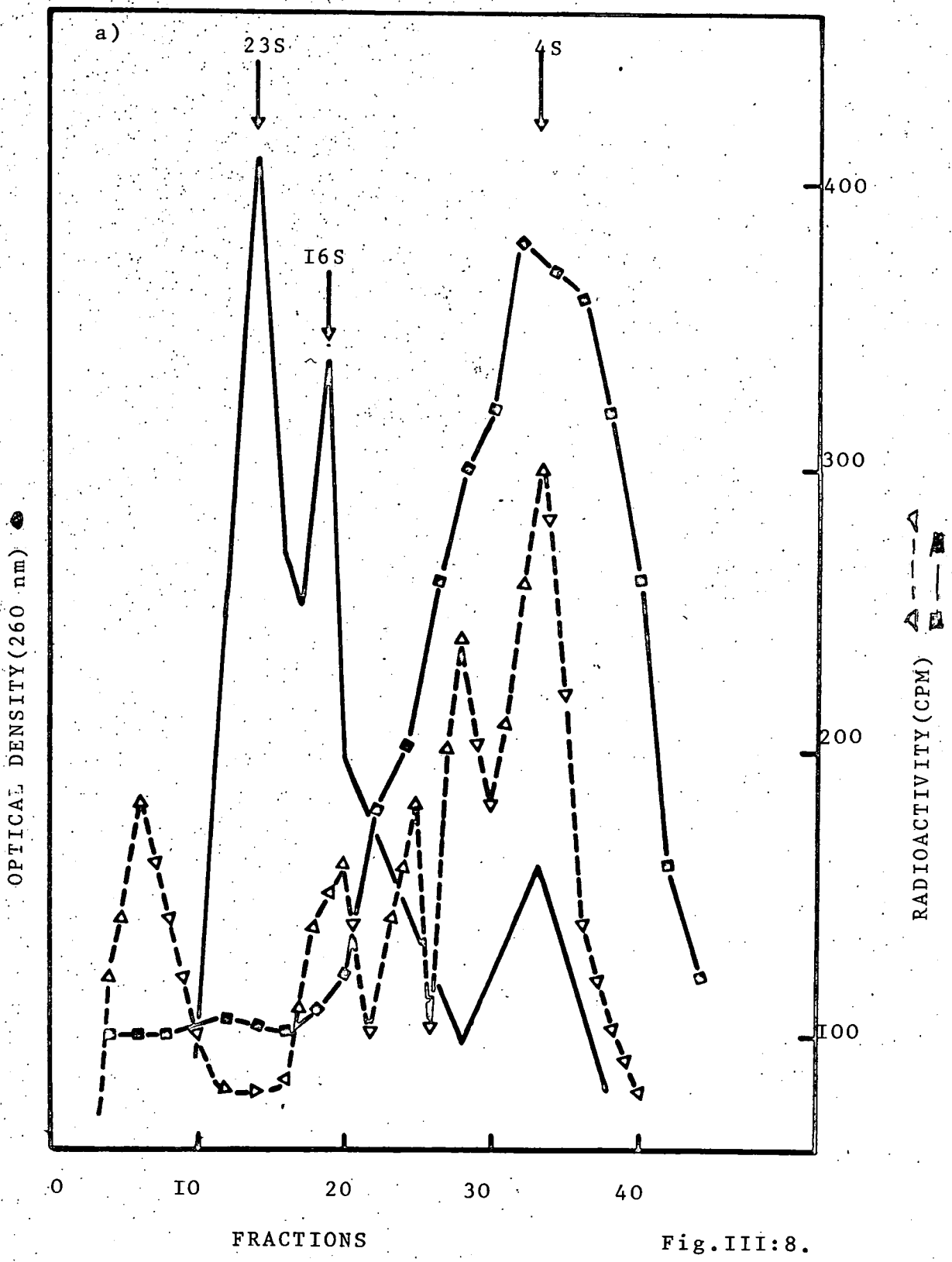

a)
23S
4S
16S
400
300
200
100
0
10
20
30
40
FRACTIONS
OPTICAL DENSITY (260 nm)
RADIOACTIVITY (CPM)

Fig.III:8.

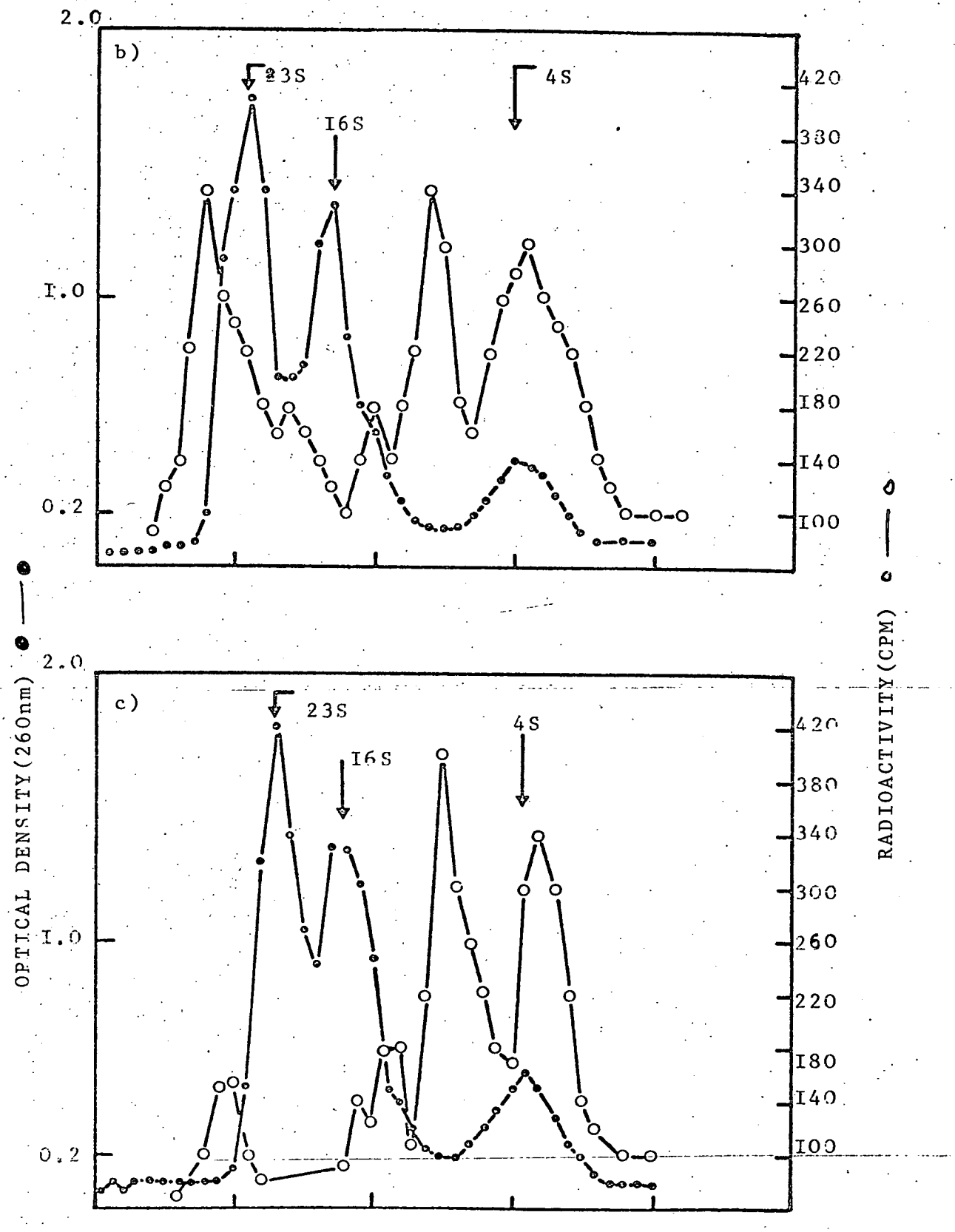

b)
23S
16S
4S
2.0
1.0
0.2
420
380
340
300
260
220
180
140
100
c)
23S
16S
4S
2.0
1.0
0.2
420
380
340
300
260
220
180
140
100
OPTICAL DENSITY (260nm)
RADIOACTIVITY (CPM)
FRACTIONS

Fig.III:8.

suggest that either they are inefficiently synthesised or are broken down by nucleases present either in the transcription reaction or the sucrose gradient.

6. Transcription of Adenovirus DNA by Micrococcus luteus DNA-dependent RNA polymerase.

This enzyme also transcribes Adenovirus DNA (Figure III:9a) but much less RNA is synthesised than with E. coli RNA polymerase.

The amount of RNA synthesised is dependent on the concentrations of cations in the incubation mix. Figure III:9b shows that RNA incorporation is maximal around 6mM Mg^{++} or around 2-3mM Mn^{++}, the Mg^{++} producing greater stimulation. These optimums are in agreement with those found for the action of M. luteus RNA polymerase on Calf Thymus DNA (Fox and Weiss, 1964) and Xenopus rDNA (Hecht, 1973).

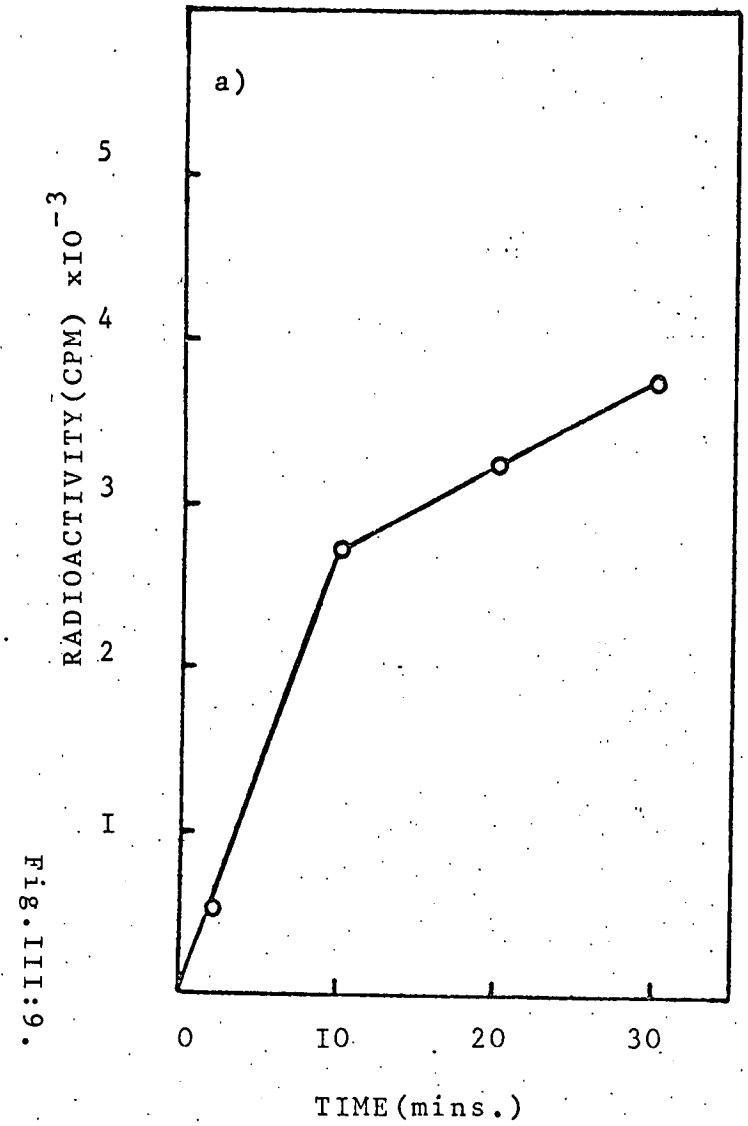
It is not clear why the M. luteus RNA polymerase should be less efficient than the E. coli RNA polymerase but it may be the result of polymerase-cRNA binding or end-product inhibition. KCl is inhibitory for the action of this polymerase in some cases (e.g. Hecht, 1973) but the concentrations used here ($\leq$ 0.1M KCl) are probably too low to account for the reduction of synthesis. Perhaps reduction is due to non-reinitiation of chain growth which is known to occur with M. luteus RNA polymerase (see Hecht, 1973; Richardson, 1969).

As a result of this decreased activity with the M. luteus RNA polymerase, all subsequent experiments involving Adenovirus cRNAs utilised the E. coli RNA polymerase.

Conclusions

There are three main conclusions which can be drawn from the

Figure III:9. Transcription of Adenovirus 2 DNA by M.luteus DNA-dependent RNA polymerase.a) Incubation mix same as for Figure III:5a(0.09M Kcl) but 2.5 units M.luteus enzyme.Same sample volumes also taken as for FigureIII:5a. There is approximately IO times less incorporation with the M.luteus enzyme as compared to the E.coli enzyme.b) incubation mix as for Figure III:6. RNA TCA precipitated after IOmins. incubation at 37^{o}C. o-o $MgCl_2$;x-x $MnCl_2$.


a)
RADIOACTIVITY (CPM) xIO^{-3}
5
4
3
2
I
0
IO
20
30
TIME (mins.)

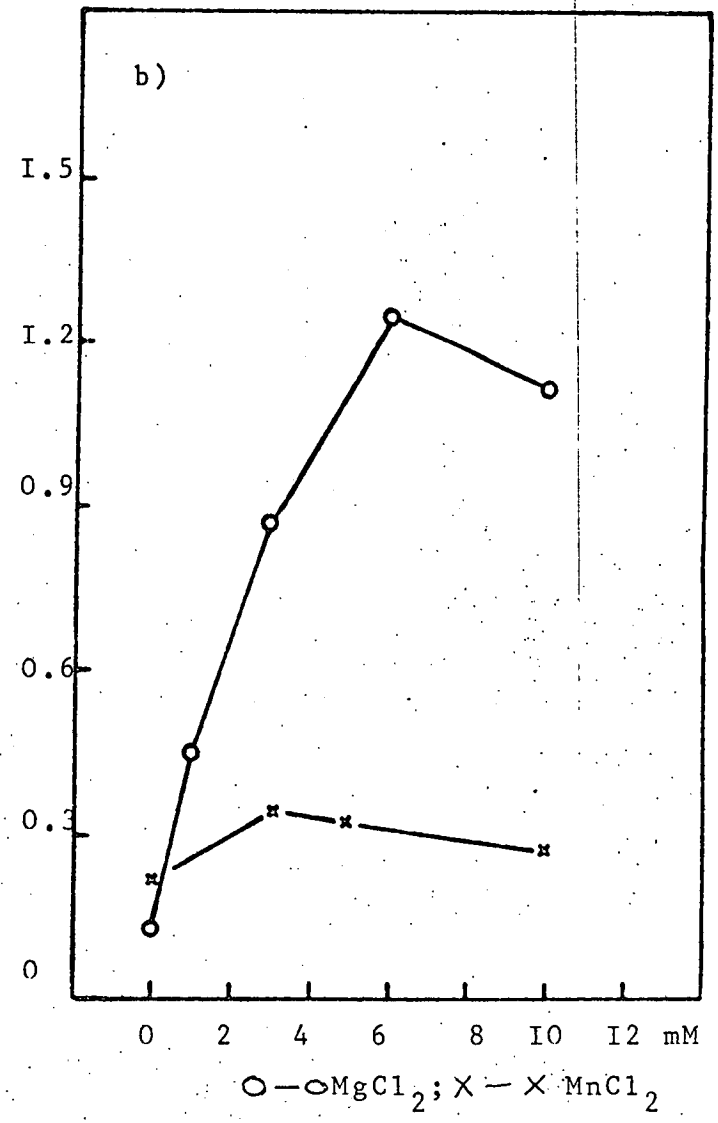

b)
I.5
I.2
0.9
0.6
0.3
0
0
2
4
6
8
IO
I2 mM
O—O MgCl$_2$; X — X MnCl$_2$

Fig. III:9.

results of the experiments described above. First, Adenovirus cRNA synthesis using the E. coli DNA-dependent RNA polymerase, does not appear to be special or unique in that the reaction dependencies are similar to those found for a variety of other E. coli RNA polymerase-DNA interactions. This, in effect, means that peculiar conditions of synthesis do not have to be met before Adenovirus DNA can be transcribed efficiently. Secondly, the transcription reaction can be somewhat optimised for the production of RNA by considering the various influences that have been described above. The most optimal conditions of synthesis are summarised in Table III:3. Finally, together with these considerations, the above experiments suggest that Adenovirus DNA may be preferentially transcribed in vitro since the size of the cRNAs is markedly heterogeneous and no transcripts approaching the molecular length of one complete DNA strand are observed. This suggestion is borne out by the results of subsequent experiments described in the following section.

Table III:3

Incubation mixes for transcription of Adenovirus DNA by E. coli DNA - dependent RNA polymerase

$MgCl_2$+$MnCl_2$		$MgCl_2$ alone		$MnCl_2$ alone	
Tris pH 7.5	100mM	Tris pH 7.5	100mM	Tris pH 7.5	100mM
$MnCl_2$	0.5-2mM	$MgCl_2$	4-12mM	$MnCl_2$	4mM
$MgCl_2$	4mM	Spermidine	1.6mM	Spermidine	1.6mM
Spermidine	1.6mM	KCl	0.2M	KCl	0.2M
KCl	0.1M	K_2HPO_4	2mM	K_2HPO_4	2mM
K_2HPO_4	2 mM	Enzyme	2.5 units	Enzyme	2.5 units
Enzyme	2.5 units	DNA	1-2μg	DNA	1-2μg
DNA	1-2μg	nTPs	5n moles each	nTPs	5n moles each
nTPs	5n moles each				

SECTION III

CHARACTERISATION OF ADENOVIRUS cRNAs

It has been known for a long time that nucleic acid hybridisation techniques can be useful for the detection of specific nucleic acid base-sequence homology (e.g. McCarthy and Church, 1970; Walker, 1969; Bishop, 1972b). Many features of the process contribute to the preciseness and specificity obtained and these are outlined in a brief account.

An important consideration in molecular hybridisation studies is characterisation of the RNA species. Failure to characterise the RNA, or the hybrids, can frequently lead to misinterpretations of results, or the failure of the technique to detect regions of nucleic acid homology which would otherwise be apparent. This last point is particularly important when trying to detect sequences represented in small amounts within individual genomes. Some of the evidence on virus DNA sequences in certain eukaryote cells suggests that they exist in small amounts (see Table I:1). Since the Adenovirus cRNAs are the probes used to measure the amount of virus DNA sequences in these virus-exposed cells, it was considered particularly desirable to characterise them. The results of this section show that such characterisation is essential before virus DNA in transformed or tumour cells can be quantitated. In the following section use is made of cRNA and DNA excess hybridisation which are now both described.

Native DNA can be dissociated and reassociated *in vitro* (Marmur and Lane, 1960; Doty *et al.*,1960) and hybrid duplex DNA molecules can be formed from the DNA of two different bacterial species (Schildkraut *et al.*, 1961), viruses (Schildkraut *et al.*, 1962; Sutton, 1972) or higher organisms (Walker, 1969; Hoyer, McCarthy and Bolton, 1964; McCarthy and Church, 1970; Kohne, 1970). Similarly, specific complex-formation between denatured and complementary RNA can be brought about by the process of annealing at high temperature and high salt concentration (Hall and Spiegelman, 1961; Schildkraut, Marmur, Fresco and Doty, 1961) and such hybridisation between complementary nucleic acids can be considered to be very highly specific (McCarthy and Church, 1970; Walker, 1969; Bishop, 1972b).

There are, essentially, two types of hybridisation experiments; RNA excess and DNA excess. In the second type of reaction the proportion of the RNA which is complementary to the DNA can be measured, and the rate constant of the reactive species can be estimated (Melli *et al.*, 1971; Bishop *et al.*, 1972; Bishop, 1972a).

In RNA excess experiments, unlike DNA excess experiments where the reactants are usually in solution, the DNA is usually immobilised on nitrocellulose membrane filters so as to prevent DNA reassociation. (Nygaard and Hall, 1964; Gillespie and Spiegelman, 1965). Both DNA-DNA reassociation and RNA-DNA hybridisation can behave as second order reactions (Nygaard and Hall, 1964; Wetmur and Davidson, 1968; Young and Paul, 1973), and the rate of the reactions are influenced, for the most part, by similar factors.

Nygaard and Hall (1964) have shown that the initial rate of hybridisation is proportional to the initial RNA concentration, and

Bishop (1969) has demonstrated that the rate of RNA-DNA hybrid formation, throughout the reaction, is still a function of the initial RNA concentration. Further, if the RNA concentration, throughout the reaction, is well in excess of the complementary DNA sequence then the rate of hybridisation, roughly, is inversely proportional to the genetic complexity of the RNA (Bishop, 1969; Birnstiel et al., 1972; Purdom et al., 1972). In this respect DNA-RNA hybridisation is closely analogous to DNA-DNA reassociation where the rate is inversely proportional to the base sequence complexity of the DNA (Britten and Kohne, 1968; Wetmur and Davidson, 1968). Conveniently, for RNA excess hybridisation reactions, the rate of RNA-DNA hybrid formation can be derived from the double-reciprocal plot of hybridisation versus time (Bishop, 1969); and Birnstiel et al. (1972) have used the term $Crt\frac{1}{2}$ (the product of initial RNA concentration, in moles/nucleotide/liter^{-1}, and the time necessary to reach half-saturation, in secs.), to measure kinetic complexities of RNA species in individual hybridisation reactions. This term ($Crt\frac{1}{2}$) is reasonably analogous to $Cot\frac{1}{2}$ (the product, in moles nucleotide/litre^{-1}/sec., of initial DNA concentration and the time taken to reach half-reassociation) which has been introduced by Britten and Kohne (1968) to help characterise DNA-DNA reassociation reactions.

There are other parameters which affect the rate of DNA-DNA reassociation, some of which influence the rate of RNA-DNA hybridisation. The maximum rate of reassociation generally occurs at a temperature of 20-30^{o}C below the melting temperature (Tm) of the DNA (Marmur and Doty, 1961; Wetmur and Davidson, 1968); the reaction

rate increases slightly with the GC content of the DNA (Wetmur and Davidson, 1968); higher salt concentrations give a faster rate (Schildkraut and Lifson, 1965; Wetmur and Davidson, 1968; Britten, 1969); and the rate is proportional to the square root of the molecular weight of the DNA (Wetmur and Davidson, 1968) or exceptionally is inversely proportional to the fragment size of the DNA (Hutton and Wetmur, 1973; Chilton, 1973). The reassociation rate is also inversely proportional to the solvent viscosity (Wetmur and Davidson, 1968), and mismatching can reduce the overall reaction rate, although by how much is not certain. Hutton and Wetmur (1973b) estimate that the reaction rate of deaminated DNA is reduced by a factor of 2 when there is 33% mismatch in the final duplex, and glyoxalated DNA with 16% mismatch reduces the renaturation rate also by a factor of 2. Bonner et al. (1973) using deaminated DNA and also renaturation of interspecies bacterial DNAs, estimate the reduction in reaction rate due to mismatching to be almost twice that of Hutton and Wetmur (1973b); and a far greater dependence on mismatching on reaction rate has been proposed by Sutton and McCallum (1971) and Sutton (1972).

For RNA-DNA hybridisation the dependencies are less clear. In general there is a marked dependence on temperature (Birnstiel et al., 1972; Bishop, 1972b) and the rate increases with increased salt concentration (Bishop, 1972a). Whether there is any effect of GC content, RNA molecular length, or mismatching on the rate of the hybridisation reaction is not precisely known. There is some evidence that for RNA excess hybridisation reactions there is no length dependence (Birnstiel et al., 1972), whereas for I:1 RNA:DNA

reactions the rate is proportional to the molecular length (Hutton and Wetmur, 1973b) and for DNA excess reactions the rate may be inversely proportional to the fragment size of the DNA at least (Bishop, 1972b).

In general, if the hybridisation reaction is carried out under conditions of RNA excess (Young and Paul, 1973; Bishop, 1972b) and the RNA consists of a single reactive species - or a few species all at the same concentration - the reaction is second order and the rate of approach to saturation (and the double-reciprocal transformation) can be used to measure the kinetic complexities of the individual RNA species. In addition, if the hybridisation reaction is carried out under the same criteria every time, then the kinetic complexities of a variety of RNAs can be compared. These kinetic complexity values can be related to the analytical complexities of the RNAs studied.

The rate of hybridisation in DNA excess experiments is also a function of analytical complexity (Bishop, 1972; Bishop, 1972b), and in addition, can provide information on the repetition frequency of the DNA sequences complementary to the RNA.

Accordingly, cRNAs were synthesised from Adenovirus DNA templates using the E. coli DNA-dependent RNA polymerase (see this Chapter, Section II; and Materials and Methods). These cRNAs were then hybridised to their homologous template DNAs in conditions of RNA excess or DNA excess. From the RNA excess experiments, double-reciprocal linear plots were constructed for individual reactions, and the saturation values and $Crt\frac{1}{2}s$ determined. Using these values, and standards, the kinetic complexities of the virus cRNAs were obtained. The fidelity of transcription was also examined.

Results and Discussion

1. Effect of RNase on Adenovirus DNA-cRNA hybrids

Some DNA-RNA hybrids are sensitive to pancreatic RNase digestion. For example, the enzyme partially degrades homologous hybrids formed between *E. coli* rRNA and *E. coli* DNA (Yankofsky and Spiegelman, 1962), λcRNA and λ DNA (Green, 1970), and trytophan messenger RNA and several bacterial DNAs (Denney and Yanofsky, 1972). Bishop (1972b) has also commented on the susceptibility of certain RNA-DNA hybrids to RNase digestion; bacteriophage T4 and *P. mirabilis* cRNAs only hybridise to between 60 and 75% of their expected saturation values. Equally, saturation values can be apparently increased by failure to use the appropriate enzymatic concentrations.

Adenovirus cRNAs were hybridised to their homologous DNA templates and the resulting hybrids treated with varying concentrations of pancreatic RNase. The results, for Adenovirus 12 cRNA-12 DNA hybrids are shown in Figure III:10. At concentrations of around 10μg/ml RNase the hybrids appear to be resistant to digestion. Similar results were obtained for Adenovirus 2 and Adenovirus 7 cRNA-homologous DNA hybrids (data not shown) and increasing the time of digestion did not lower the hybridisation values obtained with 10μg/ml. for 20 minutes incubation. The saturation values, in addition, are in agreement with the findings of the subsequent kinetic experiments so that there does not seem to be appreciable digestion of fully-formed cRNA-DNA hybrids. For subsequent experiments involving the use of RNase, concentrations of 10μg/ml. were used.

Figure III:IO. Effect of RNase(pancreaticA) on Adenovirus I2cRNA-DNA hybrids. Various concentrations of RNase(made up in 2% Na acetate pH5 and diluted in 2xSSC) were added to Adenovirus I2cRNA-DNA hybrids on membrane filters.Hybridisation was performed in 6xSSC 30%FA with Adenovirus I2 cRNA(S.A. $I.7xIO^{7}$cpm/ug) in a IO:I excess over the DNA(5ng Adenovirus DNA plus 2ug M.luteus DNA as carrier/filter).The reaction was carried out at $50^{o}C$ for 20% of the final saturation value. o-o Adenovirus I2cRNA-DNA hybrids treated once with different concentrations of RNase.x--x Adenovirus I2 cRNA-DNA hybrids treated once with RNase at different concentrations and then a second time with IOug/ml in 2xSSC.

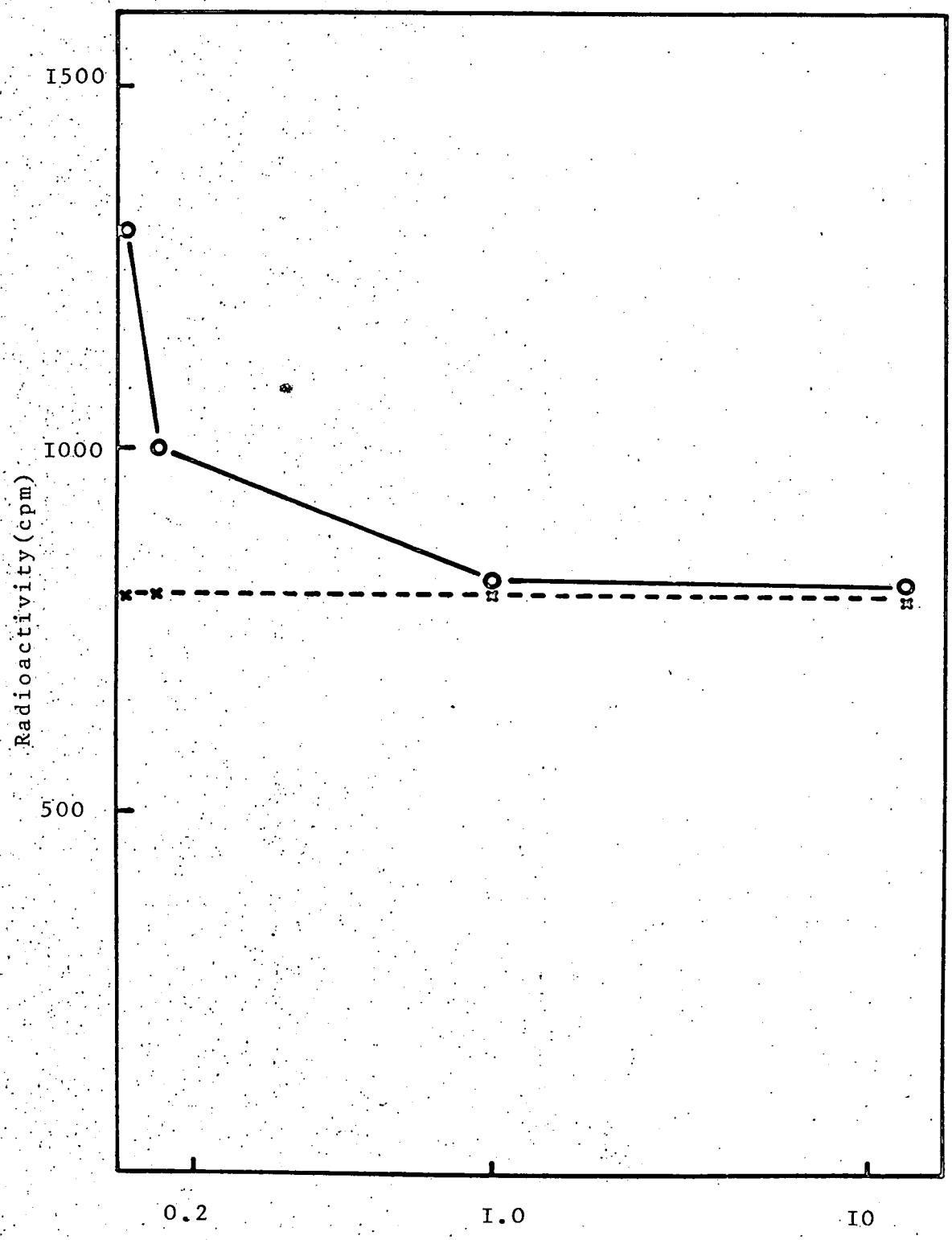


Fig. III:10.

2. Optimum rate temperatures for hybridisation of Adenovirus cRNAs

Initial rates of cRNA-DNA hybrid formation for Adenovirus cRNA-DNAs were determined by hybridising cRNAs to their homologous DNAs to less than 50% of the final saturation value, in conditions of cRNA excess. The temperature optimums (T.OPT.) in 6 x SSC/30% Formamide (FA) and 1 x SSC were obtained, and are shown in Figure III:11 and Table III:4. The lowering of the T.OPT. values due to the Formamide is consistent with the findings of McConaughy et al. (1969); that a 1% increase in Formamide leads to a 0.7°C decrease in thermal stability of nucleic acid helices. Also, the effect of Na^+ ion concentration is in agreement with the increase in T.OPT. for renaturation with increasing salt concentration (Schildkraut and Lifson, 1965). The T.OPTs for the three virus cRNAs are very similar: Adenovirus 12, 2 and 7 being 51°C, 52°C and 53°C respectively in 6 x SSC 30% FA.

The Tms of these virus DNAs are higher by some 30°C than the T.OPT. values in the same salt (Table III:4). RNAs with known high GC contents appear to have closer Tm^{DNA}/T.OPT. hybrid differences. For example, Xenopus ribosomal 28 and 18S RNA (GC content, 59%) has a 9°C difference in 6 x SSC/50% FA (Birnstiel et al., 1972); and E. coli cRNA (GC content, 50%) has a difference in 3 x SSC/50% FA, of around 11°C (Bishop, 1972a). The Tm^{DNA}/T.OPT. hybrid difference for RNAs of lesser GC content appears to be greater; Ø x 174 DNA (GC content, 42%) has a Tm of 64°C in 6 x SSC 50% FA and the cRNA a T.OPT. in the same solution, of 45°C giving a difference of 19°C (Birnstiel et al., 1972); while P. mirabilis cRNA (GC content, 39%) has a Tm^{DNA}/T.OPT. hybrid difference also of around 19°C (Bishop, 1972a).

Figure III:II. Optimal rate temperature for the formation of Adenovirus cRNA-DNA hybrids. Carried out in 6xSSC 30%FA with cRNA(S.A. $I.7xIO^{7}$ cpm/ug) at 2:I excess over DNA on filters(5ng each). Reactions were terminated after IO-20% of the final saturation value and the hybrids treated as described(Materials and Methods,pg. 38-39). 2ug carrier M.luteus DNA was added to each filter before hybridisation and the background radioactivity due to this subtracted.
x-x Adenovirus I2cRNA;o-o Adenovirus 7cRNA;o--o Adenovirus 2cRNA.

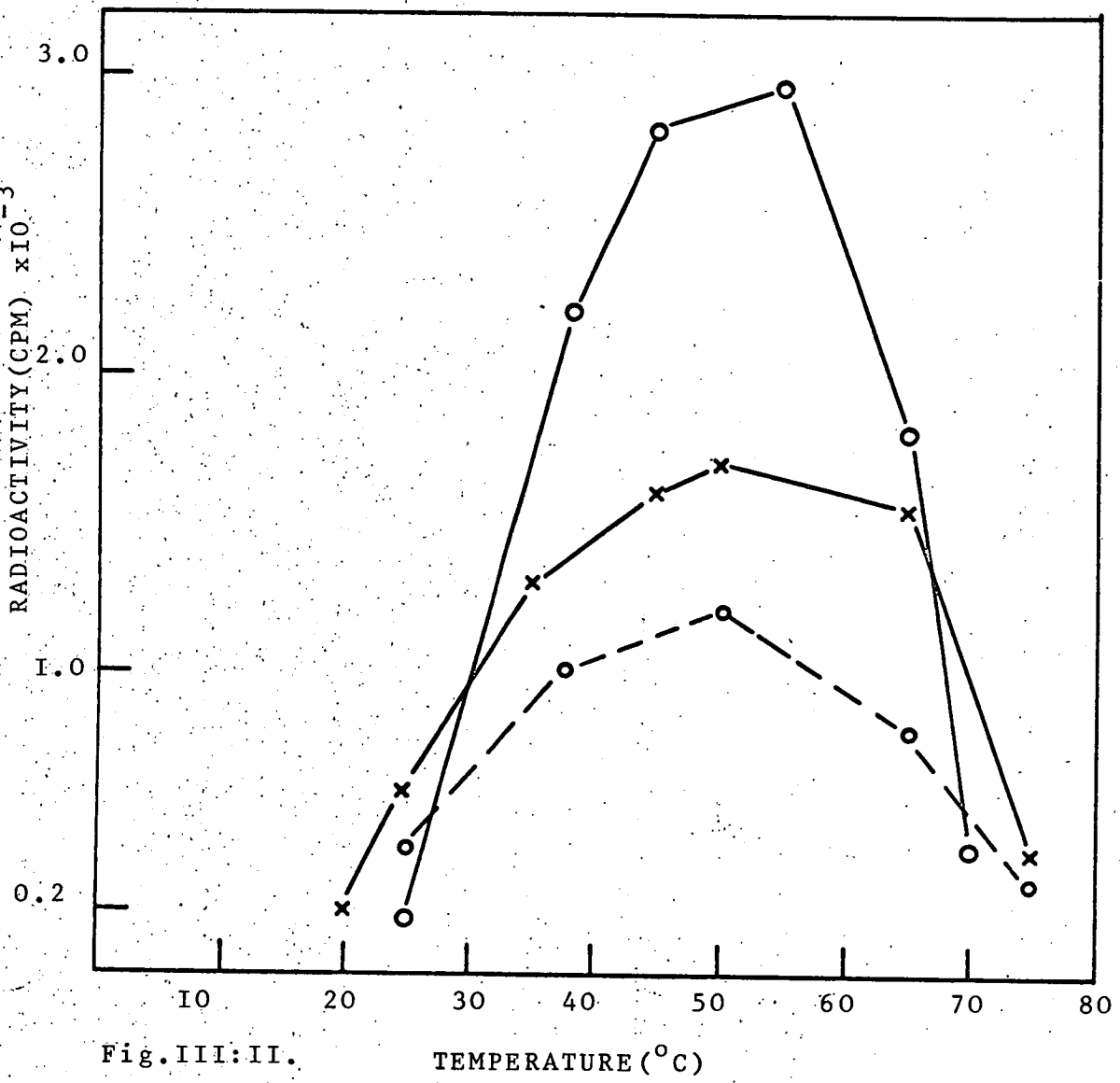

3.0
2.0
1.0
0.2
RADIOACTIVITY (CPM) xIO^{-3}
10
20
30
40
50
60
70
80
TEMPERATURE (^{o}C)

Fig.III:II.

Table III:4

virus cRNA	T.OPT. 6xSSC/30% FA	T.OPT. 1xSSC	Tm^{hybrid} 1xSSC	Tm^{DNA}* 1xSSC	$Tm^{hybrid} - T.OPT.^{h}$ 1xSSC	$Tm^{DNA} - T.OPT.^{h}$ 1xSSC	$Tm^{DNA} - Tm^{h}$ 1xSSC
Adeno-12	51	60	72	89.0	12	30	17.0
Adeno-7	53	63	75	90	12	27.0	15
Adeno-2	52	61	78	92.5	17	31.5	14.5

* See Table III:1

AT-rich satellite cRNAs have T.OPTs which are 30-40°C lower than the Tms of the native DNAs (see Section IV, this Chapter). In general, then, the lower the GC content of the RNA in an RNA-DNA hybrid, the greater the Tm^{DNA}/T.OPT.^{hybrid}/difference. Figure III:12 reflects this finding. What is clearly evident is the discrepancy between the GC contents of Adenovirus DNAs and the Tm^{DNA}/T.OPT hybrid differences. This is especially true for Adenovirus 2 cRNA, the corresponding DNA duplex having a GC content of 58% (Green, 1970; Table III:1, this Thesis) which is approximately 10% higher than Adenovirus 12 or 7 DNAs. That the T.OPT. values for the virus cRNA-DNA reactions are low suggests that regions of low GC content may be preferentially hybridising. Subsequent experiments lend additional support to this suggestion.

3. Base-sequence complexity and rates of hybridisation of Adenovirus cRNAs

Figures III:13, III:14 and III:15 show double-reciprocal linear plots for the hybridisation of Adenovirus cRNAs to their template DNAs. The reactions were all carried out in RNA excess conditions, at the T.OPT. for each cRNA in 6 x SSC/30%FA, the Formamide being considered useful in maintaining the molecular weight of the RNA during long incubation times (Birnstiel et al., 1972; McConaughy et al., 1969). The $t\frac{1}{2}$s for the individual reactions were determined, and the $Crt\frac{1}{2}$s calculated. These $Crt\frac{1}{2}$s are presented in Table III:5. Each of the Adenovirus cRNA-DNA hybridisation reactions has its own $t\frac{1}{2}$, and the complexity of each RNA is consequently different. The kinetic complexity of Xenopus ribosomal RNA (28 and 18S) was determined under the same criteria (see below) and this value together with a

Figure III:I2. Variation in Tm DNA/T.OPT. RNA-DNA hybrid formation with GC content for a variety of DNAs and their corresponding hybrids.In general,as the GC content becomes lower for the DNA,the TmDNA/T.OPT. becomes larger.Note the position of Adenovirus 2 cRNA-DNA hybrid/T.OPT./Tm DNA in relation to its GC content. See text for details.

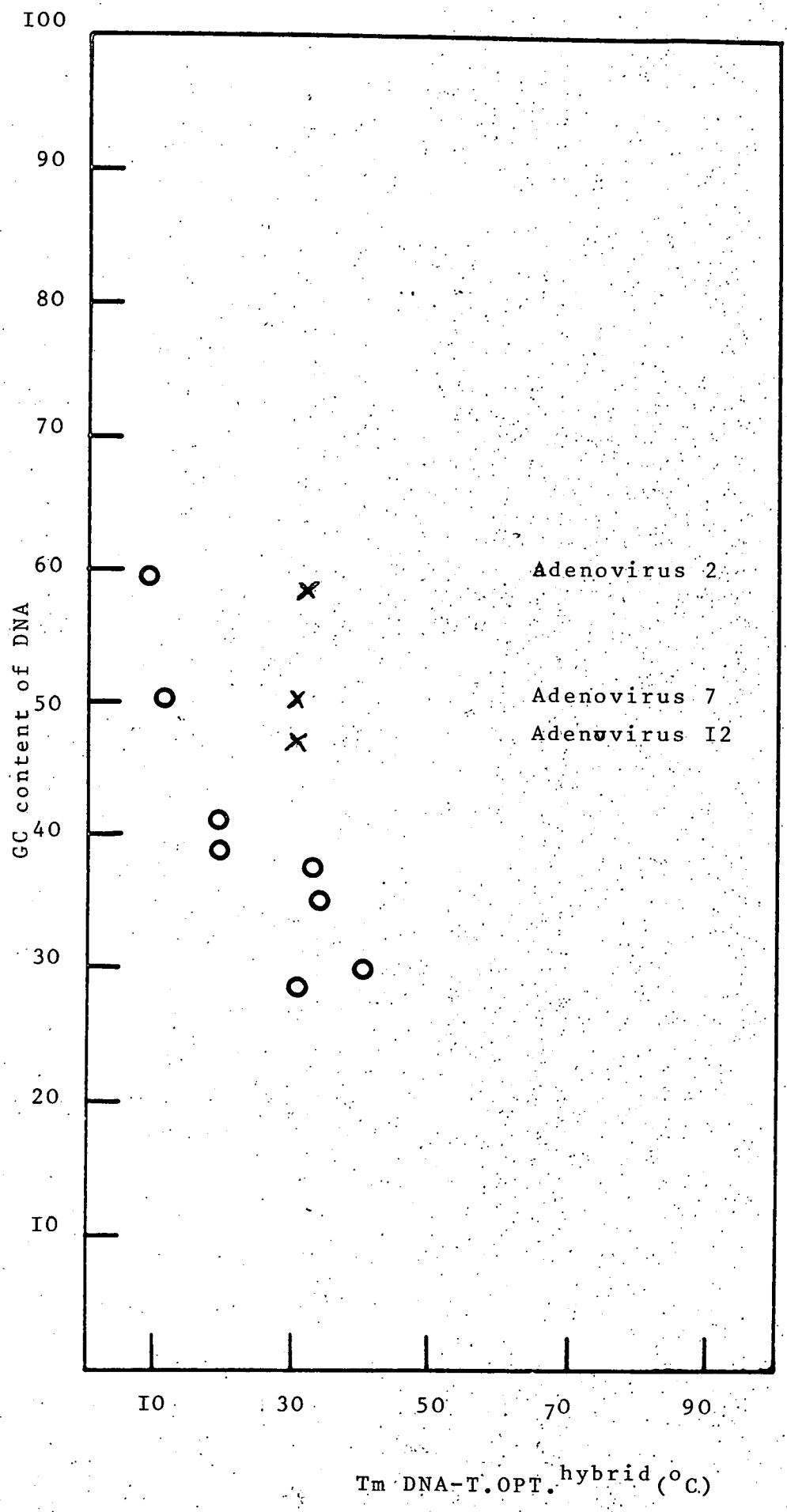

100
90
80
70
60
50
40
30
20
10
GC content of DNA
Adenovirus 2
Adenovirus 7
Adenovirus 12
10
30
50
70
90
Tm DNA-T.OPT. hybrid (°C)

Fig.III:12.

Ø x 174 cRNA standard (Sinsheimer, 1959; Birnstiel et al., 1972) allowed comparisons for the individual virus cRNAs to be made.

4. Kinetic Complexity of Xenopus ribosomal RNA

28 and 18S Xenopus ribosomal RNA was prepared as described in Materials and Methods. Figure III:16 demonstrates that this RNA preparation was homogeneous for ribosomal RNA base sequences since it exclusively hybridised to DNA sequences of ribosomal GC content ($1.724\ gm/cm^{-3}$). A hybridisation experiment was then carried out in order to derive the kinetic complexity of this RNA population. The result is shown in Table III:5. The kinetic complexity of 1.9 is the same as previously reported by Birnstiel et al. (1972).

Although the RNA consists of a population of 18S and 28S RNA molecules, the kinetic complexity can be considered as a mean value (Birnstiel et al., 1972), the individual 18S and 28S RNA molecules actually having their own kinetic complexities.

5. % DNA Complementary to Adenovirus cRNAs.

The saturation values for different Adenovirus cRNA-DNA reactions are shown in Table III:5. All these values are low, and represent only a percentage of the possible saturation value. For example, Adenovirus 12 cRNA hybridises only to 20% of the complementary DNA sequences, and Adenovirus 7 cRNA hybridises to only approximately half this amount of the available complementary DNA sequences. These low saturation values suggest that the virus cRNAs, in the main, represent only a proportion of their individual template DNA. Moreover the saturation values for each virus cRNA are in agreement with the $t\frac{1}{2}$s for each hybridisation reaction.

Figure III:I3. Hybridisation of Adenovirus 2 cRNA to Adenovirus 2 DNA immobilised on membrane filters.Reaction carried out at T.OPT.(see Figure III:II) in 6xSSC 30%FA with DNA at 5ng/filter and cRNA(S.A. I.7 $xI0^7$cpm/ug) at calculated 2:I excess over total DNA sequences.M.luteus DNA carrier(2ug/filter) was also added. The $t_{\frac{1}{2}}$ for the reaction is calculated from the double reciprocal linear plot (Bishop,I969;Birnstiel et al.,I972) by obtaining the time value at which the reaction is half complete. Standardised $t_{\frac{1}{2}}$s(3ug/ml) are presented in Table III:5.

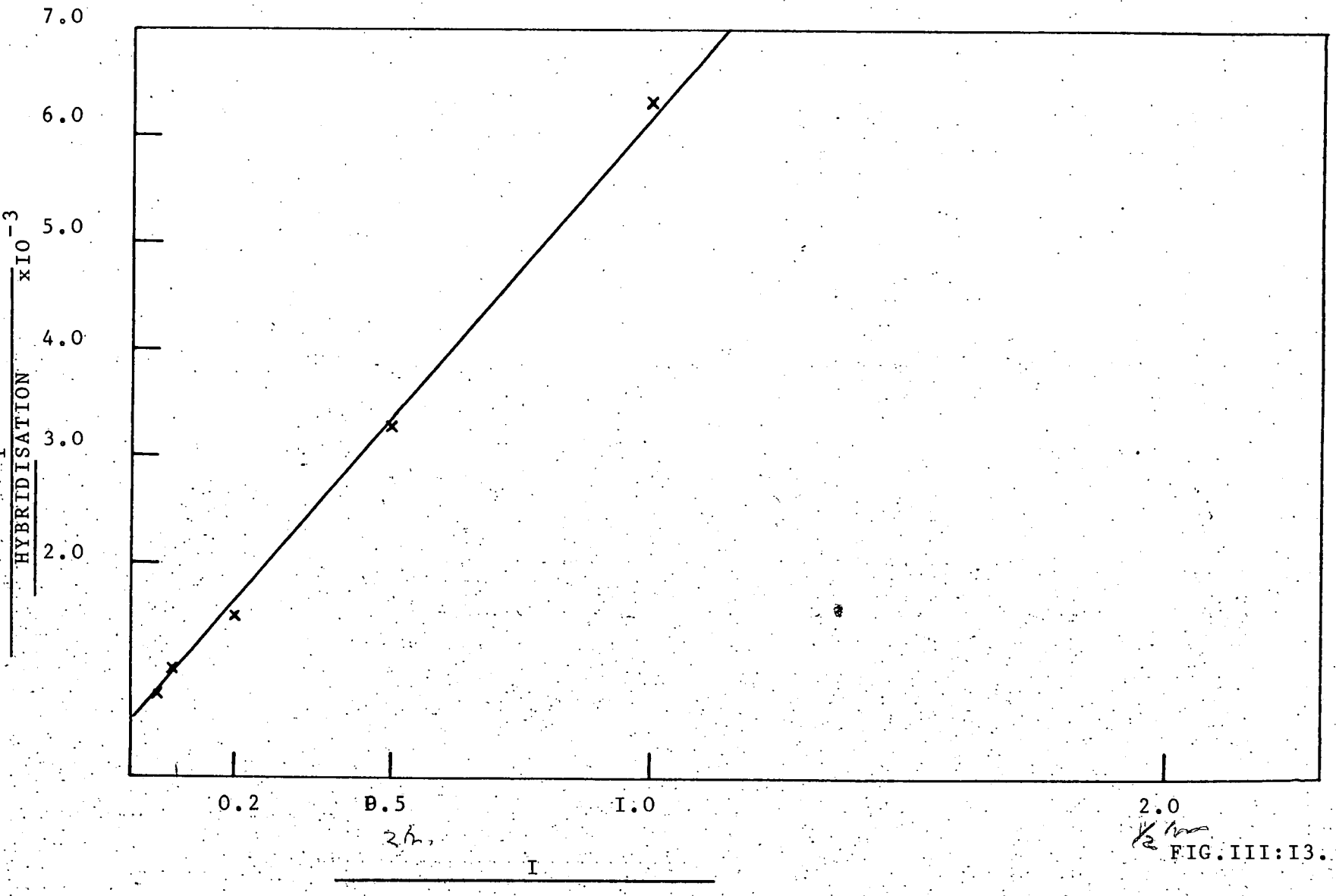


FIG. III:13.

Figure III:I4. Hybridisation of Adenovirus 7cRNA to Adenovirus 7 DNA on filters. Data expressed as double reciprocal linear plot(Bishop,I969).Conditions of hybridisation as for Figure III:I3 and III:I5.

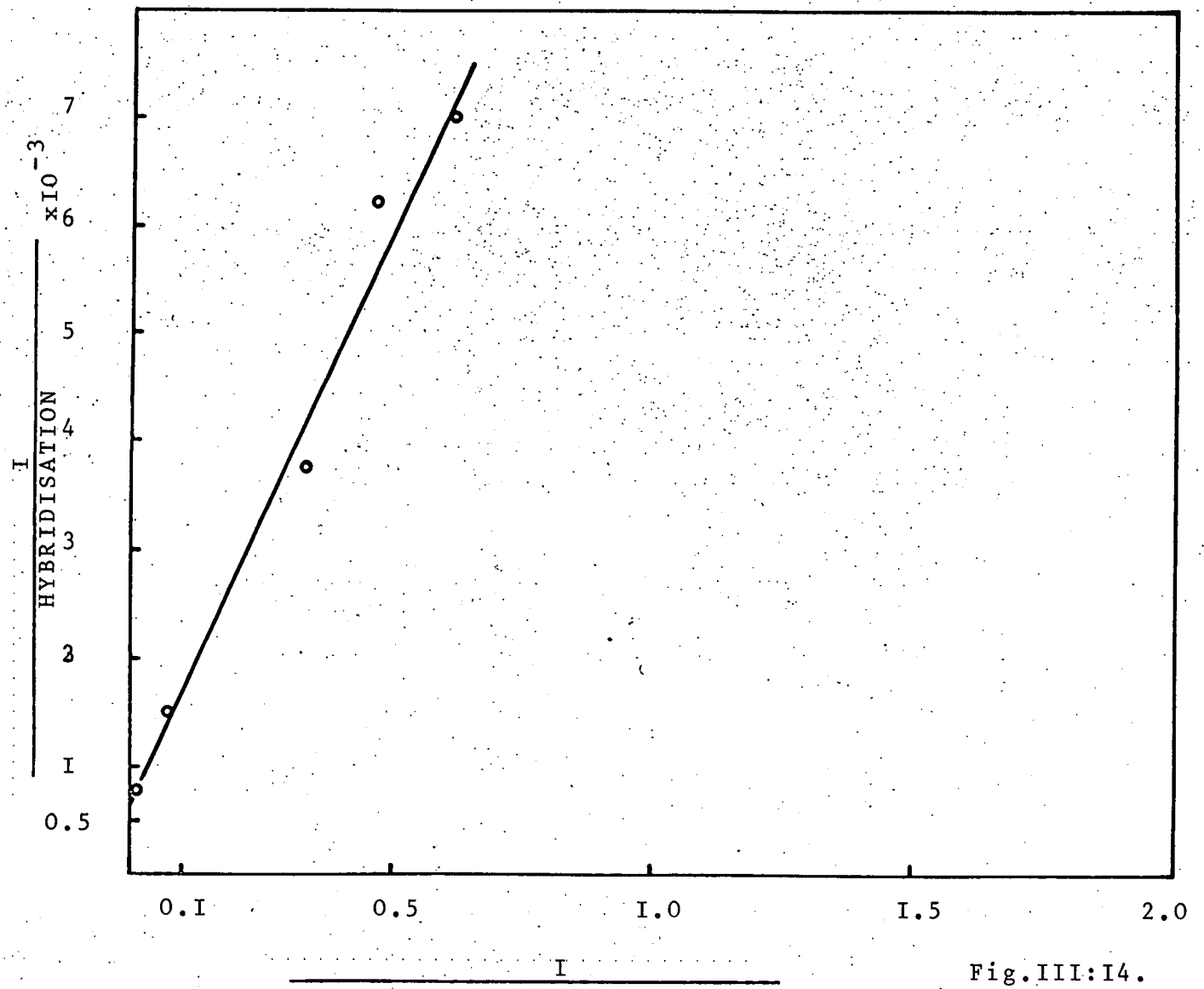

$\frac{1}{\text{HYBRIDISATION}} \times 10^{-3}$
7
6
5
4
3
2
1
0.5
0.1
0.5
1.0
1.5
2.0
$\frac{1}{\text{TIME (Hours)}}$

Fig.III:14.

Figure III:I5. Hybridisation of Adenovirus I2 cRNA to Adenovirus I2 DNA over time periods. Conditions of hybridisation are the same as presented in FigureIII:I3.

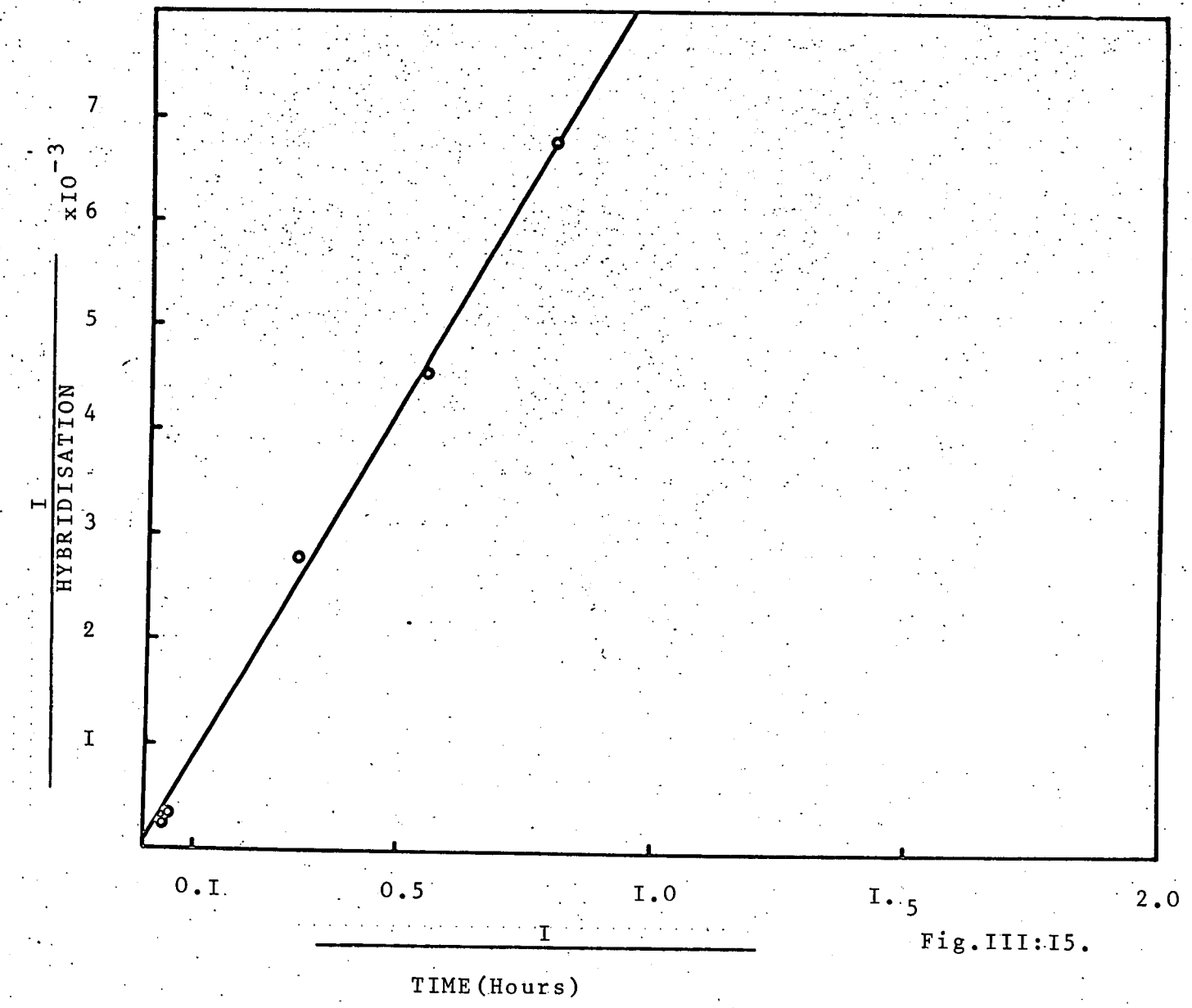


Fig.III:I5.

Figure III:I6. Hybridisation of 28 and I8S rRNA(Xenopus kidney) to Xenopus DNA fractionated on a neutral CsCl gradient.rRNA(S.A. $2xIO^5$cpm/ug) ;6xSSC 30%FA at 70^oC and the reaction stopped after IO hours incubation.Hybridisation is to DNA sequences with a buoyant density of I.724 gm/cm^{-3}which is the buoyant density of Xenopus rDNA sequences in neutral CsCl (Birnstiel et al.,I970).
M.luteus DNA(I.73Igm/cm^{-3})as density marker.

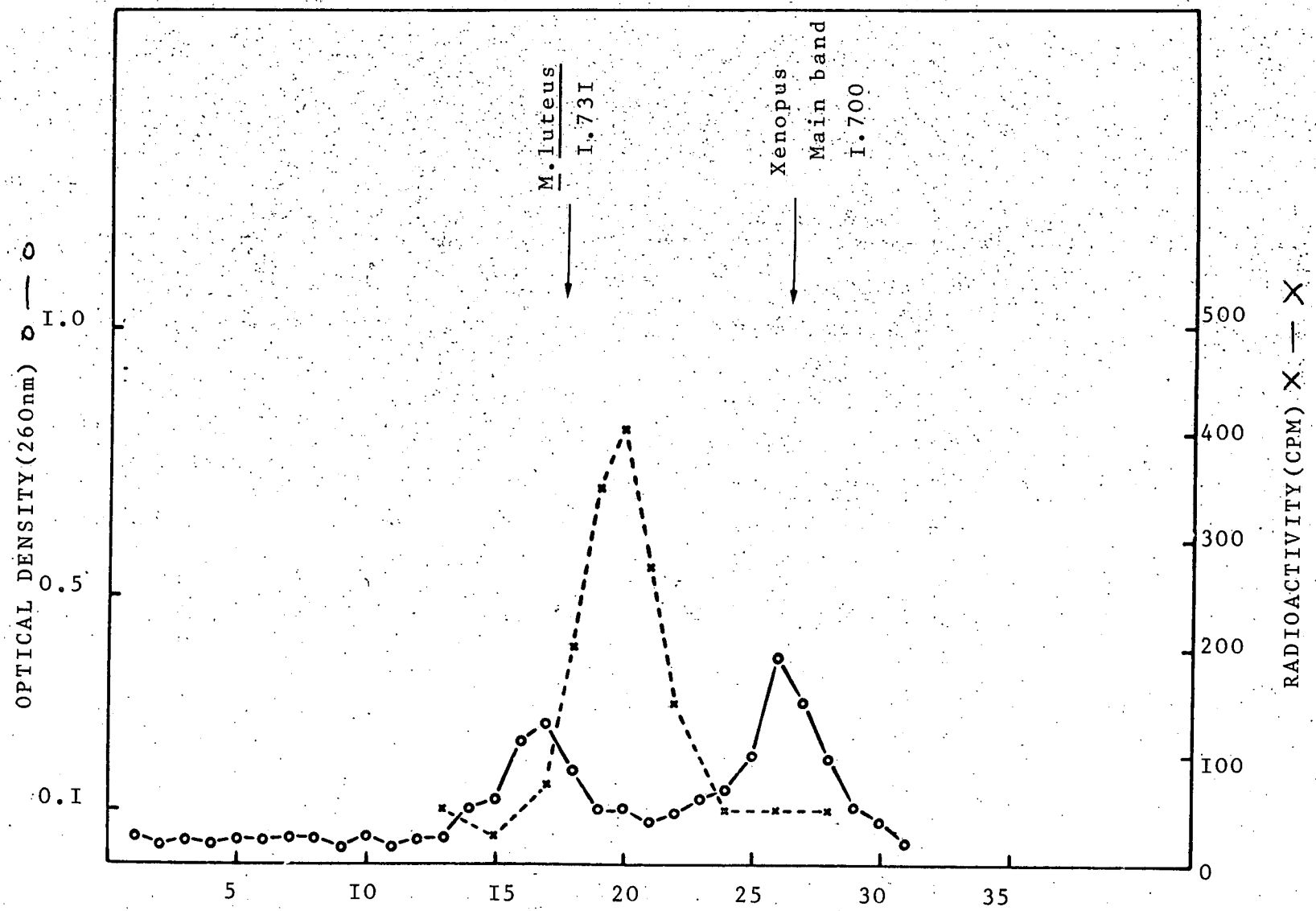

M. luteus
I.731
Xenopus
Main band
I.700
OPTICAL DENSITY (260nm) o — o
RADIOACTIVITY (CPM) x — x
I.0
0.5
0.I
500
400
300
200
I00
0
5
I0
I5
20
25
30
35
CsCl FRACTIONS.

Fig.III:I6.

Adenovirus DNA has an analytical complexity of around 25 x 10^6 daltons (Green, 1970), which is the amount of deoxyribonucleotides in the virus genome. If the cRNA from an individual virus serotype represented a total asymmetric transcript then the molecular length of this cRNA would be in the region of 1.25 x 10^7 daltons. ø x 174 cRNA (1.6 x 10^6 daltons) and Xenopus ribosomal RNA (28S and 18S) (2.2 x 10^6 daltons) possess $t\frac{1}{2}$s (3µg/ml) of 24-30 minutes (Birnstiel et al., 1972; Table III:5, this Thesis). This means that for an RNA complexity of approximately 1.25 x 10^7 daltons, and under the same conditions of hybridisation, around 300 minutes would be needed before the reaction would be at half-saturation. The $t\frac{1}{2}$ for Adenovirus 12 cRNA is around 30 minutes which is approximately 10% of the above value, suggesting that the cRNA that is hybridising is around 10 times less complex than the equivalent of one total transcript of the Adenovirus genome. Similarly, the $t\frac{1}{2}$ values for Adenovirus 2 and Adenovirus 7 represent the hybridisation of RNA sequences which are a lot less complex than one total transcript of the virus genome (Table III:5).

The DNA of Adenovirus 12 appears to be transcribed by the E. coli RNA polymerase, to a greater extent than the other two virus DNAs. Adenovirus 2 DNA has a high GC content (58-59%) and apparently is the DNA to be least transcribed by the E. coli RNA polymerase. It has already been shown that AT-rich base-sequences in Adenovirus 2 cRNA appear to be preferentially annealing in a homologous hybridisation reaction (Figure III:11, III:12). The results of the kinetic experiments show that certain cRNA sequences are preferentially hybridising and furthermore, these sequences are most likely

preferentially transcribed *in vitro*.

The double-reciprocal linear plot, however, is not strictly applicable to hybridisation reactions involving a population of RNA molecules which are all present at different concentrations. That is, the linear plot essentially represents the hybridisation of RNA sequences which are present in a large concentration in the RNA population. RNA sequences which are present in very low concentrations in the reaction are unlikely to be detected by RNA excess hybridisation reactions unless these sequences themselves are in excess of the complementary DNA sequences. Consequently the Adenovirus cRNAs could consist of a large proportion of base sequences which are transcribed from a limited region of the genome, and also a minor proportion of sequences which have been transcribed from the rest of the genome.

There are other reasons why the saturation values for the Adenovirus cRNAs might be low. First of all, the cRNA could conceivably consist of virus-specific RNA molecules plus RNA sequences which are not complementary to the virus genome i.e. the transcription by the *E. coli* polymerase is not faithful. Secondly, the reaction might not be in cRNA excess even for the greater proportion of RNA sequences present; failure to achieve RNA excess in these types of hybridisation experiments can lead to under-estimates of saturation values (Young and Paul, 1973; Birnstiel *et al.*, 1972). Thirdly, self-complementarity of the cRNA could result in reduced saturation values, and could influence the reaction rate. Such self-complementarity of cRNA and its effect on hybridisation reaction rates, has been noted (Bishop, 1969); T4 cRNA preparations were up to 40% RNAse-

resistant due to RNA-RNA self-annealing. Finally, DNA could be lost from the filters as the reaction proceeds or the amount of DNA immobilised in the filters could be over-estimated. Various experiments were performed to test these variables; although the $Crt\frac{1}{2}$ values for each of the hybridising cRNAs are, in themselves, evidence that the majority of the cRNA sequences represent only a proportion of the Adenovirus genomes.

6. Self-Complementarity of Adenovirus cRNAs.

Adenovirus cRNAs were self-annealed in 6 x SSC/30% FA at the T.OPT. at which each Adenovirus cRNA hybridised. The reactions were terminated after an equivalent of several $t\frac{1}{2}$s in the hybridisation reaction. The RNAse-resistance is shown, for each Adenovirus cRNA, in Table III:6 together with the self-complementarities of cRNAs transcribed from denatured virus templates. The cRNAs transcribed from native DNA templates show little self-annealing that would interfere with cRNA-DNA hybridisation; the cRNAs to denatured Adenovirus DNA template, however, show considerable RNAse-resistance. This high level of RNAse-resistance in the latter case is in agreement with the loss of asymmetric transcription when the template DNA of a variety of organisms is denatured during a transcription reaction (Richardson, 1969; Chamberlin & Berg, 1962).

7. Effect of RNA concentration on the saturation value and $Crt\frac{1}{2}$.

For the membrane procedure (Gillespie and Spiegelman, 1965) the rate of hybrid formation throughout the entire reaction is a simple function of the initial RNA concentration (Bishop, 1969), and by multiplying the concentration of the input RNA (Cr;moles/nucleotide/l^{-1}) and $t\frac{1}{2}$(sec.) for independent reactions with different initial RNA

Table III:5

RNA	Complementary DNA[o] (%)	$t_{\frac{1}{2}}$(3ug/ml) (mins)	$Crt_{\frac{1}{2}} \times 10^{3}$ (moles.sec./l.)	Approx. Kinetic Complexity (daltons x 10^{-6})
In 6 x SSC/30% F.A.				
Adeno-12 cRNA	20	30'	16	1.9
Adeno-7 cRNA	10	15-20'	11	1.1
Adeno-2 cRNA	5-10	12-15'	8	0.9
Xenopus rRNA (28 & 18S)	0.075	30	16	1.9
Ø x 174 cRNA[a]	95	24-27	15	Kinetic Standard

[a] Taken from Birnstiel et al. (1972).

[o] Double-stranded.

Table III:6

Self-complementarity of cRNA

RNA	% RNase-resistance (10 t 1/2s)
Adenovirus 2	10-15
Adenovirus 7	11-15
Adenovirus 12	5-7
[a]Adenovirus denatured 2	25-30
[a]Adenovirus denatured 7	20-22
[a]Adenovirus denatured 12	42-44

[a] DNA was denatured previous to transcription with *E. coli* RNA-polymerase by boiling at 100°C for 15 minutes in 0.001 m NaCl.

concentrations, a constant $Crt\frac{1}{2}$ value should be obtained (Birnstiel et al., 1972; Purdom et al, 1972). Table III:7 illustrates a nearly constant $Crt\frac{1}{2}$ value for each of the Adenovirus cRNA-homologous DNA hybridisation reactions.

Actually there is a slight rise in the $Crt\frac{1}{2}$ value, for each Adenovirus cRNA, at very high cRNA inputs which suggests that there may be sequences in the cRNA which are under-represented at low RNA inputs; although even at these relatively low RNA inputs the reaction is still being carried out under RNA excess conditions for the majority of the RNA sequences. Theoretically if the hybridisation reactions were not being carried out in cRNA excess and the cRNAs represented a single concentration of complete genome transcripts, then the reaction rate would be very slow. Young and Paul (1973) have demonstrated that decreasing an RNA:DNA ratio from approximately 10:1 to 1:1 results in about a 30% decrease in the reaction rate. As has already been pointed out, the $t\frac{1}{2}$s for individual virus cRNA-DNA hybridisation reactions are lower than would be expected from the theoretical expectations if the cRNA represented complete Adenovirus genome transcripts. If the reactions were not in RNA excess conditions, then the kinetic complexities of the viral cRNAs would in fact be lower and not higher than those obtained already. Thus for the majority of sequences in the different cRNAs, the reactions are in RNA excess.

8. The base sequences in Adenovirus cRNAs are all viral-specific

Adenovirus cRNAs were hybridised to their homologous DNAs in conditions of DNA excess. The hybridisation of Adenovirus 12 cRNA to Adenovirus 12 DNA is shown in Figure III:17; the % RNAse-resistance being plotted against log Cot (Britten and Kohne, 1968; Melli et al., 1971; Bishop, 1972a). The hybridisation value, for this particular

Table III:7

cRNA	[a] Initial cRNA concentration (μg/ml)	$Crt\frac{1}{2} \times 10^3$ (moles/sec/litre)
Adenovirus 12	0.05	16
"	0.5	16
"	0.7	18
Adenovirus 2	0.05	8
"	0.5	9
Adenovirus 7	0.05	11
"	0.5	11
"	0.7	12

[a] Hybridisation conducted at the T.OPT. for individual reactions in 6 x SSC 30% FA (see Table III:4)

cRNA, is around 80% and the $Cot\frac{1}{2}$ is 0.1. Bacteriophage λ cRNA also hybridises, in DNA excess, to its template DNA with a $Cot\frac{1}{2}$ of 0.1 (Bishop, 1972a). The analytical complexity (genome size) of λ DNA is 3×10^7 daltons (Bishop, 1969) and its GC content is 49%. The GC content of Adenovirus 12 DNA is 49% also (Green, 1970) and it has an analytical complexity of around 2.5×10^7 which is near the genome size of λ DNA. The $Cot\frac{1}{2}$ values obtained in the same salt (2 x SSC) and under the same incubation temperatures ($70^{\circ}C$), for both these virus DNAs are therefore in excellent agreement, both experimentally and theoretically.

The $Cot\frac{1}{2}$ for the reassociation of Adenovirus 12 DNA can be calculated from the $Cot\frac{1}{2}$ hybridisation value of 0.1. Bishop (1972a) has shown that, in general, the $Cot\frac{1}{2}$ reassociation value is approximately 3 times faster than the $Cot\frac{1}{2}$ value for hybridisation in DNA excess hybridisation experiments. This gives a $Cot\frac{1}{2}$, for the reassociation of Adenovirus 12 DNA in 2 x SSC at $70^{\circ}C$, of 3.3×10^{-2}. The hybridisation values and the $Cot\frac{1}{2}$s for the other Adenovirus cRNAs are presented in Table III:8. Adenovirus 7 cRNA has a similar $Cot\frac{1}{2}$ to Adenovirus 12 cRNA. Adenovirus 2 cRNA, however, has a larger $Cot\frac{1}{2}$, its value being 0.17. Adenovirus 2 DNA has a GC content of 58-59% (Green, 1970; also Table III:1 of this Thesis). Annealing sequences with approximately 60% GC content under the same incubation conditions as annealing sequences of approximately 50% GC content increases the $Cot\frac{1}{2}$ hybridisation value by a factor of 1.5 however (Bishop, 1972a). This is due to the GC contribution. Hence the $Cot\frac{1}{2}$ hybridisation value for Adenovirus 2 cRNA will be about 1.5 times higher than expected due to the higher % of GC content in the corresponding DNA. The

actual $Cot\frac{1}{2}$ is then 0.113 which is approximately the same as obtained for the other Adenoviruses studied. Furthermore, the calculated $Cot\frac{1}{2}$ for the DNA-DNA reaction is close to the value of 1.7×10^{-2} (3 x SSC) obtained by SI nuclease monitoring (see pg. 151 of this Thesis).

The DNA excess experiments are therefore a true representation of the reassociation of Adenovirus DNA. In these experiments the excess ratio was 1000:1. For ratios of 100:1 the RNase-resistance was less than 40% (Table III:8). Since an excess ratio of 100:1 is usually sufficient to bring excess DNA-RNA reactions theoretically to completion (Bishop, 1972a; 1972b), this result suggests that the virus cRNA is probably complementary to only a percentage of the genome. By increasing the excess ratio to 1000:1 (Figure III:17; Table III:8) the DNA sequences complementary to the cRNA will be increased. Hence the 80% value. Increasing the excess ratio even further (2 times) does not increase the hybridisation value (not shown).

The remaining % of cRNA which appears to be RNase-sensitive could be the result of failure to achieve high enough DNA excess for a few sequences in the cRNA; or it could be due to slight degradation of the hybrids (Bishop, 1972b) although this is unlikely (see Figure III:10). Even less likely, some cRNA could have broken down during the incubation period. The hybridisation was carried out at 70°C in 2 x SSC so that some cRNA-DNA hybrids might be unstable at this temperature. However experiments done at 65°C in 2 x SSC also do not reach full hybridisation of the virus cRNA (Table III:8 also). Rarely does the % RNase-resistance reach 100% in DNA excess hybridisation experiments (Bishop, 1972b; Campo, 1973).

Figure III:I7. Hybridisation of Adenovirus I2 cRNA and Adenovirus I2 DNA, in DNA excess. 2xSSC, 70°C. DNA:cRNA (S.A. I.7 xI0^{7} cpm/ug): I000:I. Molecular length of DNA:400 base-pairs; cRNA:800. Hybridisation was plotted relative to non-RNased samples and the reaction terminated after a Cot of I had been reached. cRNA-cRNA self-annealing deducted.

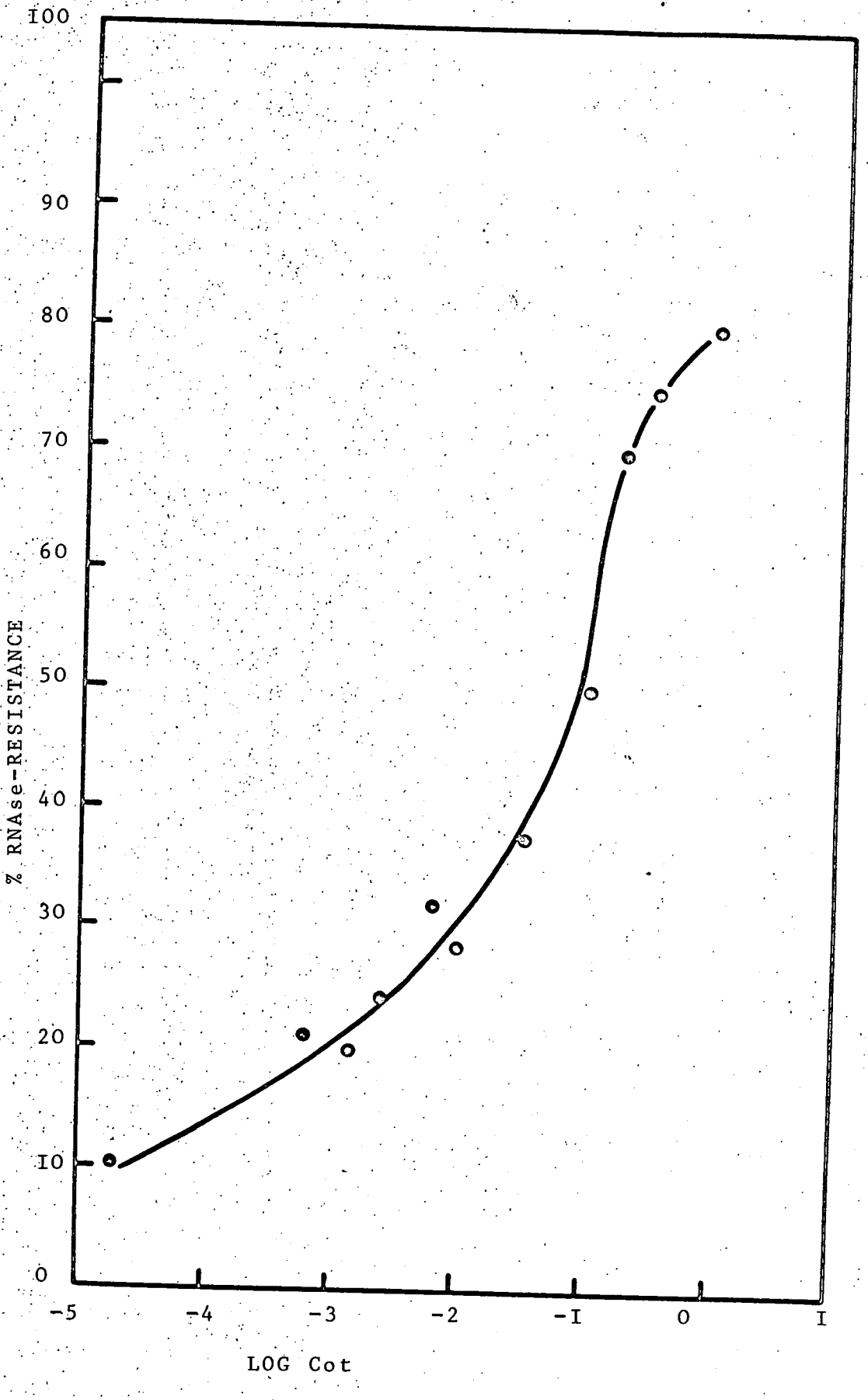

100
90
80
70
60
50
40
30
20
10
0
% RNAse-RESISTANCE
-5
-4
-3
-2
-I
0
I
LOG Cot

Fig.III:I7.

Table III:8

Cot 1/2

cRNA	DNA+	* % Hybridisation value (cpm)	$Cot\frac{1}{2}$ hybridisation	(G+C) content corrected to 50% GC	$Cot\frac{1}{2}$ DNA-DNA renaturation (calculated)
Adenovirus 12	12	80	0.1	0.1	$3.3x10^{-2}$
Adenovirus 12	12[a]	34	-	-	-
Adenovirus 2	2	75	0.17	0.113	$3.7x10^{-2}$
Adenovirus 7	7	78	0.11	0.11	$3.6x10^{-2}$
Adenovirus 12[o]	12	80	0.1	0.1	$3.3x10^{-2}$

* cRNA-cRNA subtracted (Table III:6)

a 100:1,DNA:cRNA excess

\+ 1000:1,DNA:cRNA excess

o $65^{o}C$

9. Retention of DNA on membrane filters.

Gillespie and Spiegelman (1965) demonstrated that the DNA which is immobilised on the nitrocellulose membrane filters does not become unattached during the course of an RNA excess experiment. However, when complete hybrids have been formed between homologous reacting RNA and DNA strands these hybrids can be preferentially lost from the filter. An example of this phenomenon is the loss of SV40 DNA-cRNA hybrids (Haas et al., 1972).

Accordingly, Adenovirus 2 DNA, labelled with tritium (specific activity approx. 1×10^6 cpm/μg) was loaded onto membrane filters and a hybridisation reaction performed with unlabelled Adenovirus 2 cRNA. Filters were counted before, during and after several steps in the conventional hybridisation procedure. There was no appreciable loss of DNA (data not shown). Since the cRNAs represent different concentrations of RNA sequences complementary to their template DNAs, it is unlikely that complete hybrids would be formed anyway. Further, since a loss of DNA from the filters would not affect the $t\frac{1}{2}$s for the individual cRNA-DNA hybridisation reactions, the hybridisation rate being independent of the amount of DNA on the filters (Birnstiel et al., 1972), the unexpectedly low $t\frac{1}{2}$s and low saturation values for the Adenovirus cRNA-homologous DNA reactions cannot be explained on this basis.

From the results of the above experiments it seems that the E. coli RNA polymerase is transcribing certain regions of the Adenovirus genomes more efficiently - or selectively - than others. There are two experiments which help to confirm this conclusion. First, thermal melting of the hybrids can provide information on the preciseness of base-pairing and the average GC content of the hybrid. Second, there is only a certain degree of homology between different Adenovirus serotype DNAs; 12 and 7 share 10-25% of their DNA base sequences, 12 and 2 share 20-24% of their DNA base sequences, and 7 and 2 share 24-26% of their DNA base sequences: (Green, 1970). Therefore if these common DNA sequences are not transcribed by the E. coli RNA polymerase, hybridisation reactions between cRNAs and heterologous Adenovirus DNAs will be negative.

10. Thermal dissociation of Adenovirus cRNA-DNA hybrids.

Thermal melts were performed in 0.1 x SSC. The melting profiles are shown in Figure III:18. The Tms for the individual hybrids are presented in Table III:4. They are 68°C, 62°C and 65°C in 0.1 x SSC for Adenovirus 2, 12 and 7 respectively. Green and Hodap (1972) reported similar Tms, in 0.1 x SSC-0.1% SLS, for these three Adenovirus cRNA-DNA hybrids: Adenovirus 2 (68°C), 12(64°C) and 7(66°C). In 1 x SSC the Tm for native Adenovirus 2 DNA is 92.5°C (Green and Pina, 1964; Table III:2, this Thesis) which correlates with its GC content of 58-59% (Marmur and Doty, 1962). The Tm of the Adenovirus 2 cRNA-DNA hybrid in 1 x SSC is 78°C, which is 14.5°C lower than the Tm for the native DNA. Adenovirus 12 DNA melts, in 1 x SSC, at 89°C (Green and Pina, 1964; Table III:2, this Thesis) and the corresponding hybrid dissociates with a Tm of 72°C

Figure III:I8. Thermal dissociation of Adenovirus cRNA-DNA hybrids.The hybrids were formed in 6xSSC 30%FA at the T.OPT. for individual reactions and hybridisation terminated at 80% of the reaction.After post-hybridisation treatment(see Materials and Methods,pg. 38)to remove non-specific RNA,filters were counted in Toluene-PPO, POPOP and the radioactivity determined. To melt the cRNA-DNA hybrids filters were thoroughly washed in Chloroform to remove Toluene scintillation fluid and washed in I xSSC and IxSSC containing DEP to remove Toluene and RNase activity.They were then subjected to increase in temperature while being immersed in O.IxSSC.
a) Adenovirus I2 cRNA-DNA hybrid;b) Adenovirus 7cRNA-DNA hybrid;c) Adenovirus 2 cRNA-DNA hybrid. Tm corresponds to temperature at which 50% of the hybrid has melted.

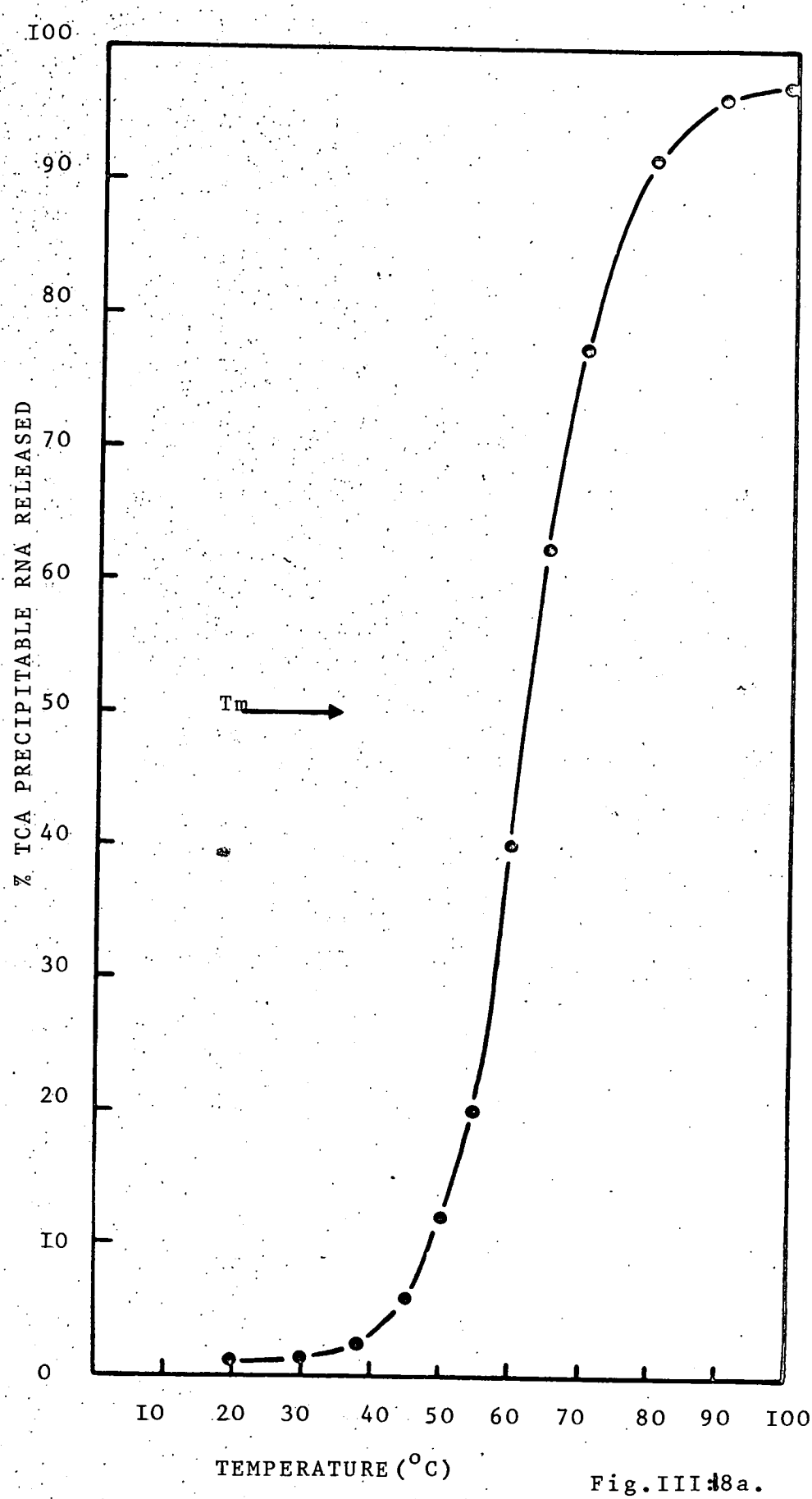
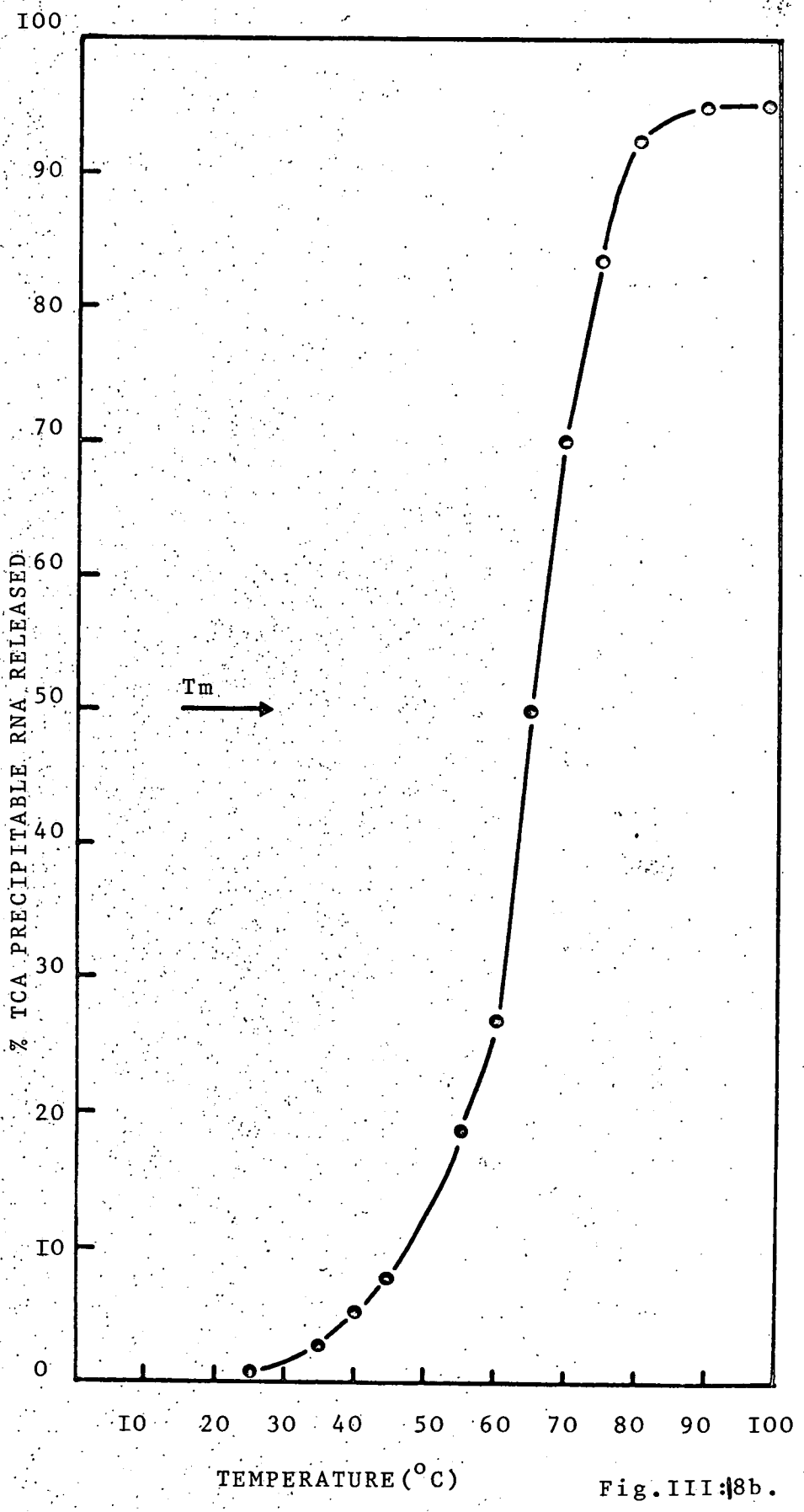

100
90
80
70
60
50
40
30
20
10
0
% TCA PRECIPITABLE RNA RELEASED
Tm
10
20
30
40
50
60
70
80
90
100
TEMPERATURE (°C)

Fig. III:8a.


100
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10
0
% TCA PRECIPITABLE RNA RELEASED
Tm
10
20
30
40
50
60
70
80
90
100
TEMPERATURE (°C)

Fig. III:8b.

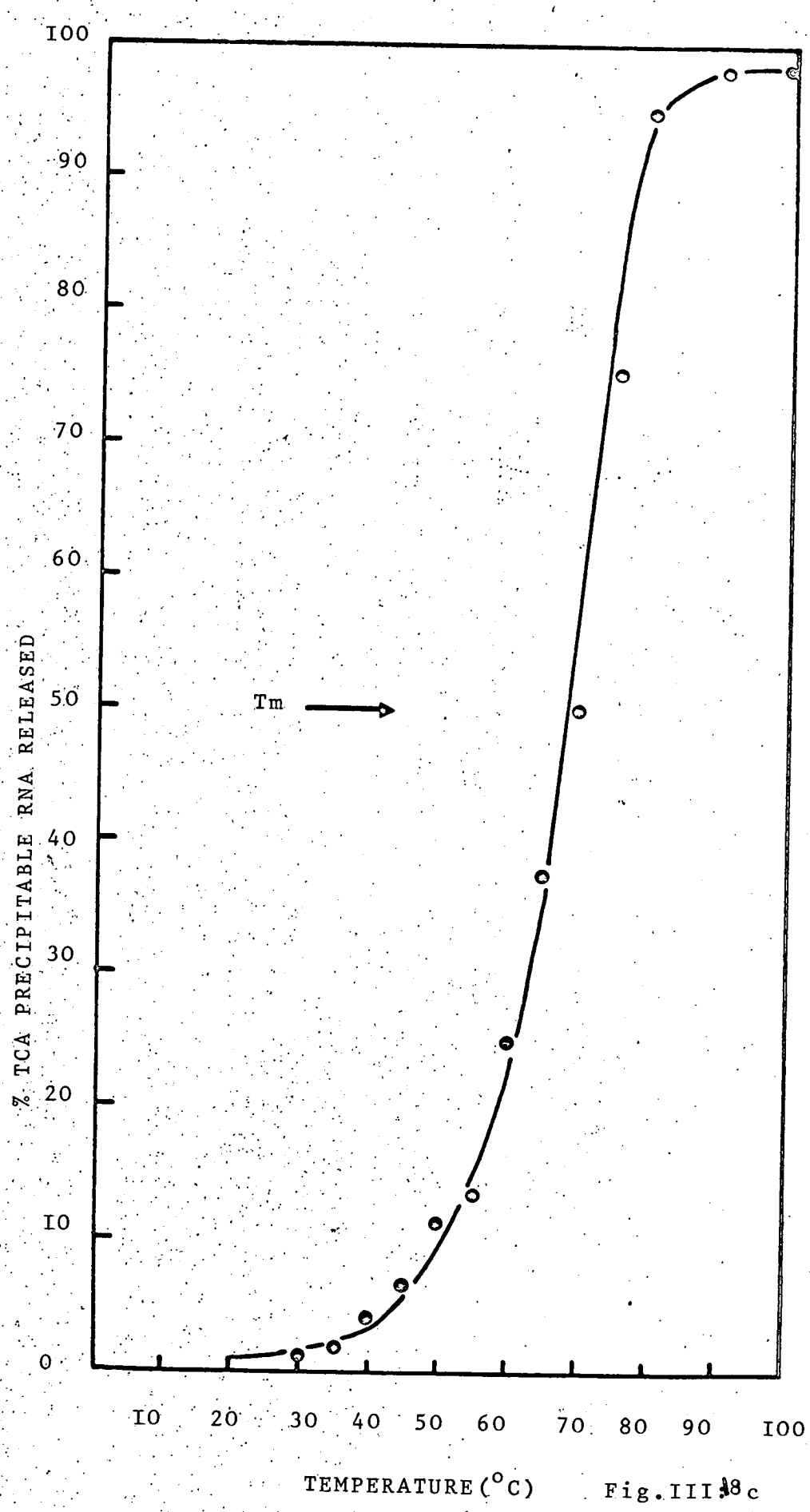

100
90
80
70
60
50
40
30
20
10
0
% TCA PRECIPITABLE RNA RELEASED
Tm
10
20
30
40
50
60
70
80
90
100
TEMPERATURE (°C)

Fig. III.18c

which is some 17°C lower. There is therefore a marked difference between the Tms of the native DNAs and the Tms of the cRNA-DNA hybrids.

The principal parameters which could contribute to the relatively low hybrid thermal stabilities are: low molecular weight RNA, low GC content, and mismatching of base pairs. For DNA-DNA renaturation studies, the expression $b\Delta T = 820^{\circ}C$ (Thomas and Dancis, 1973) connects the molecular length of a duplex(b) with the change in Tm (ΔT). For example if the single strand molecular weight of a reassociating duplex is 400 nucleotides then the Tm of the resulting duplex will be approximately 2°C lower than normal; for single strand molecular weights above 800 nucleotides this effect will be negligible. There is some evidence for this length effect occuring in RNA-DNA hybridisation reactions, either with immobilised DNA (Birnstiel et al., 1972) or with both reactants in solution (Shenkin and Burdon, 1974). However, in the experiments reported here the effect is probably minimal since a large percentage of the cRNAs possess molecular weights above 800 nucleotides (see Figure III:8b & 8c).

All the hybrids melt with a single sharp transition indicative of a high degree of precision of base pairing. There is therefore no good reason to suppose that a large percentage of mismatched pairs exist. For the hybrid Tms to be approx. 15°C lower than the native DNA Tms on the basis of mismatching alone, the hybrids would be approximately 15% mismatched (Bonner et al., 1973). This is unlikely. A likely explanation for the large temperature difference between the hybrid Tms and the native DNA Tms is that, for substantial regions, the hybrids are composed of rU-dA or rA-dT rich regions since a predominence of Uridine or Adenine residues in the ribose strand of a

mixed deoxyribose-ribose helix can markedly reduce the thermal stability (Walker, 1969). PolyrA-polydT for example has a Tm which is 4°C lower than the Tm of the corresponding polydA-polydT duplex (Chamberlin, 1965), and more strikingly, polyrU-polydA melts some 19°C below the corresponding DNA duplex with the same base composition (Walker, 1969). This suggestion would be in agreement with the low T.OPTs for the formation of the Adenovirus cRNA-DNA hybrids (Table III:4) and it is likely, moreover, that the selected transcripts of the virus genomes represent relatively AT-rich regions. It is also interesting that the majority of sequences in the Adenovirus cRNAs are common to the virus-specific RNA sequences expressed in Adenovirus transformed cells (Green, 1970; Green and Hodap, 1972). These latter sequences are of 47-51% GC content, a value which is common to several Adenovirus transformed cells including those transformed by Adenovirus type 2. In general the *in vivo* RNA is of lower GC content than the corresponding DNA (Green, 1970). Thus there may be some correlation between Adenovirus DNA transcripts *in vitro* and *in vivo*.

11. Cross-hybridisation of Adenovirus cRNAs with heterologous Adenovirus DNAs.

The human Adenoviruses can be subdivided into serotype groups on the basis of their reaction with either rat or rhesus monkey erythrocytes (Rosen, 1958; 1960); basic biochemical and immunological differences (e.g. Pereira *et al.*, 1963; Ginsberg, 1962); and oncogenicity in hamsters (e.g. Huebner, 1967). Serotypes in different groups are related in some antigenic properties (Pereira *et al.*, 1963) and by some DNA-DNA homology (Green, 1970). For Adenovirus 12 and 7

Table III:9a

cRNA[a]	DNA (5ng)	cpm hybridised*[b]	DNA(50ng)	cpm hybridised*[b]	% cross-hybridisation
Adeno-2	2	2100	2	2.2×10^4	-
Adeno-7	7	4203	7	4.08×10^4	-
Adeno-12	12	8100	12	8.02×10^4	-
Adeno-2	7	80	7	120	1%
Adeno-2	12	50	12	120	1%
Adeno-7	2	60	2	100	1%
Adeno-7	12	50	12	140	1%
Adeno-12	2	80	2	160	1%
Adeno-12	7	40	7	160	1%

[a] Always 8:1 excess (cRNA; S.A. 1.7×10^7 cpm/ug)

* 6 x SSC/30% FA, T.OPT. 2-3$t\frac{1}{2}$s.

[b] background counts for 3ug m.luteus DNA alone have been subtracted.

Table III.10

cRNA	DNA[a]	% Hybridisation value[b]
Adenovirus 12	12	80
" 2	2	75
" 7	7	78
" 12	2	15
" 2	12	6
" 7	12	16

[a] 1000:1 excess

[b] cRNA-cRNA annealing substrated (see Table III:6)

this homology extends to 25%; for 2 and 7, 26%; and for 12 and 2, 24% (Green, 1970). This level of homology has also been established and corroborated by heterology patterns in the electron microscope (Garon et al., 1973). cRNA excess and DNA excess hybridisation experiments were therefore carried out with Adenovirus cRNAs and heterologous serotype DNAs. The results are shown in Tables III:9 and III:10. Under conditions of cRNA excess (Table III:9a) there is no cross-hybridisation of Adenovirus cRNAs. Under conditions of Adenovirus DNA excess (Table III:10) there is some cross-hybridisation.

Green and Hodap (1972) also showed that cRNAs to Adenovirus DNAs do not cross-hybridise under conditions with DNA immobilised on filters. Their experiments, however, may be open to criticism. They hybridised Adenovirus 12 cRNA to Adenovirus 12 DNA and obtained 189 cpm/3 ng DNA bound (Table III:9b is taken from Green and Hodap; 1972). Taking an upper limit of 25% homology between Adenovirus 12 and Adenovirus 7 DNA (Green, 1970) around 40-50cpm/3ng would be expected for the heterologous reaction. Clearly this is a low value and may be within background range. The other homologous Adenovirus cRNA-DNA reactions give similarly low hybridisation values (Table III: 9b). Dunn et al. (1973) also reach the conclusion that Adenovirus DNAs must be selectively transcribed by the E. coli RNA polymerase since there is little cross hybridisation between different serotypes (Table III:8c). However, in this case the hybridisation reactions were not carried out under optimum conditions for the formation of these virus cRNA-DNA hybrids. On calculation their homologous reaction is only about 10% of the expected saturation value obtained in this Thesis (Table III:5). In the experiments reported here both the cRNA excess and the DNA excess experiments were carried out under

Table III:9b [adapted from Green & Hodap (1972)]

DNA on filter	DNA template for cRNA	Bound cts/min
Adenovirus 12	12	189
Adenovirus 2	12	6
Adenovirus 7	12	5
None	12	4
Adenovirus 2	2	117
Adenovirus 12	2	7
Adenovirus 7	2	7
None	2	6
Adenovirus 7	7	238
Adenovirus 12	7	6
Adenovirus 2	7	10
None	7	5

Table III:9c [adapted from Dunn et al., 1973]

DNA on membrane	Template for cRNA synthesis	cpm bound[3] to membrane
Adenovirus type 2[1]	Adenovirus type 2	5916
Adenovirus type 12	"	97
Human[2]	"	124
E. coli[2]	"	112
none	"	38
Adenovirus type 12[1]	Adenovirus type 12	8390
Adenovirus type 2[1]	"	168
Human[2]	"	132
E. coli[2]	"	100
None	"	100

1 50 ng/filter

2 50 µg/filter

3 Input 50,000 cpm/membrane

optimum conditions and the reactions were taken past saturation. Under the cRNA excess conditions there is no homology evident, while under the DNA excess conditions there is some (Table III:9a; III:10). These experiments suggest that the sequences complementary to common DNA sequences for different serotypes are under-represented in the cRNAs. However, the fact that some hybridisation occurs in the DNA excess hybridisation experiments suggests that these particular sequences are likely to be transcribed *in vitro*, although infrequently.

12. *Experiments with denatured Adenovirus DNA templates*.

Since Adenovirus DNA templates appear to be transcribed in a preferential way by the *E. coli* RNA polymerase, it was thought that denaturation of the template previous to transcription might lead to increased amounts of RNA being synthesised and loss of selectivity, there being evidence for this occurring with other denatured DNAs (Richardson, 1969).

Figure III:19 shows the T.OPTs of the respective Adenovirus cRNAs transcribed from denatured templates. In 6 x SSC 30% FA, the T.OPTs are similar: 50-53°C. The kinetic complexities of two of the Adenovirus cRNAs were determined (Figure III:20 and III:21; Table III:11), and the saturation values are shown in Table III:11. What is apparent from these results is the decreased saturation values and the concomitant increased $t\frac{1}{2}$ values for each of the Adenovirus cRNA-homologous DNA reactions compared to the homologous reactions with cRNA transcribed from a native DNA template (Table III: 5). The saturation values cannot therefore be increased by use of a denatured DNA template; a result probably due to the self-annealing of cRNA during the hybridisation reaction (Table III:6).

Figure III:I9.
Optimal rate temperature for the formation of Adenovirus cRNA-DNA hybrids, the cRNA originally being transcribed from a denatured DNA template. Conditions of hybridisation were 6xSSC 30%FA; cRNA(S.A. I.7x10^7 cpm/ug); IO:1 excess cRNA:DNA at 5ng DNA/filter. Carrier M.luteus DNA(2ug) was added to each filter, hybridisation to this DNA never exceeding 0.0I% of the homologous virus cRNA-DNA reaction.
a) Adenovirus 7b) Adenovirus I2 c) Adenovirus 2.

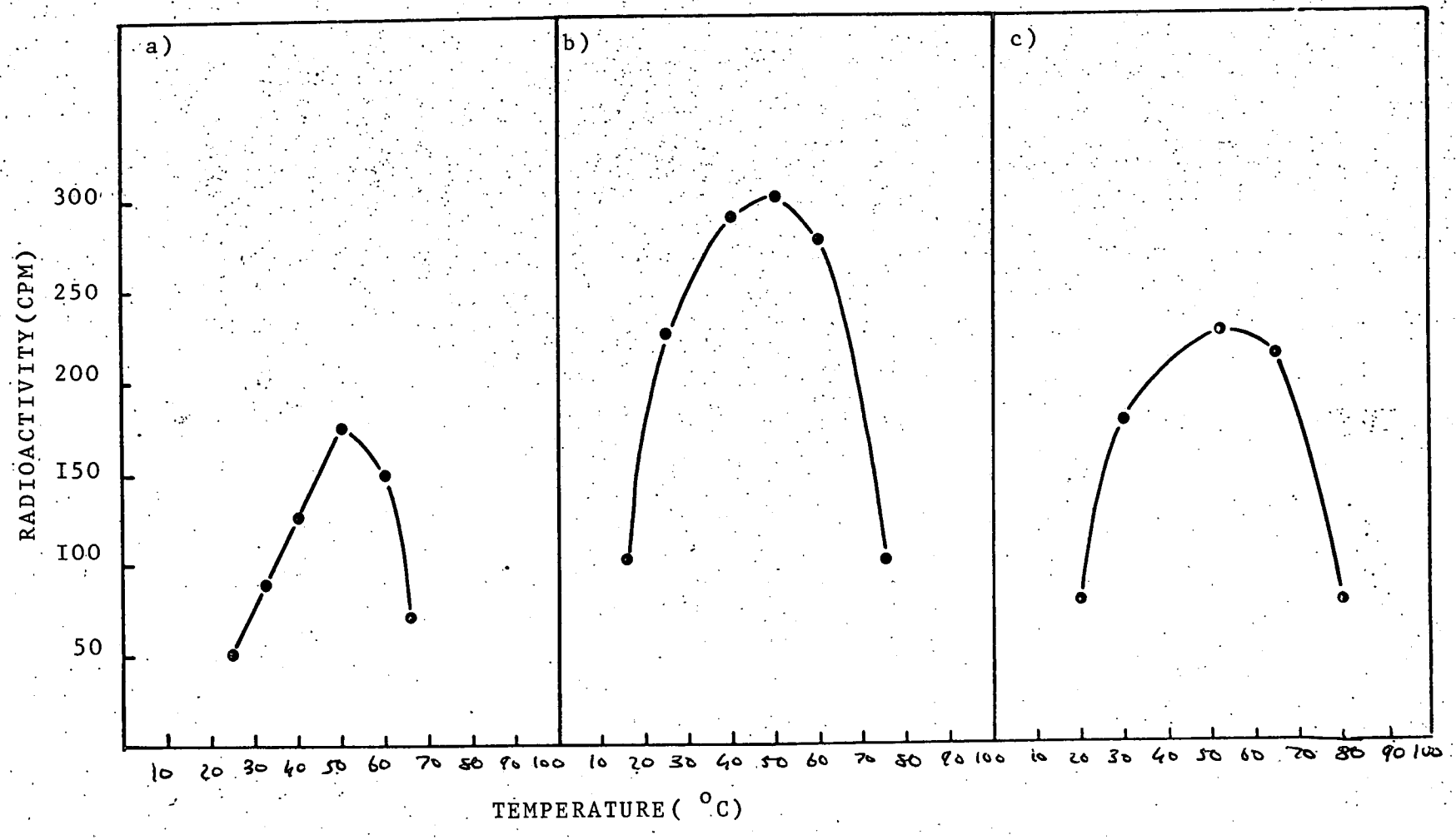

a)
b)
c)
RADIOACTIVITY (CPM)
300
250
200
150
100
50
10 20 30 40 50 60 70 80 90 100
TEMPERATURE (°C)

Fig.III:19.

Figure III:20. Hybridisation of Adenovirus 7 cRNA synthesised off a denatured template to Adenovirus 7 DNA immobilised on membrane filters. Hybridisation at T.OPT.(see Figure III:I9) in 6xSSC 30%FA with 5ng virus DNA and 2ug carrier *M.luteus* DNA on filters. cRNA(S.A. $I.7xIO^7$ cpm/ug) added at 2:I excess. The $t_{\frac{1}{2}}$ for this reaction can be calculated from the time to reach half-saturation.(see Table III:II)

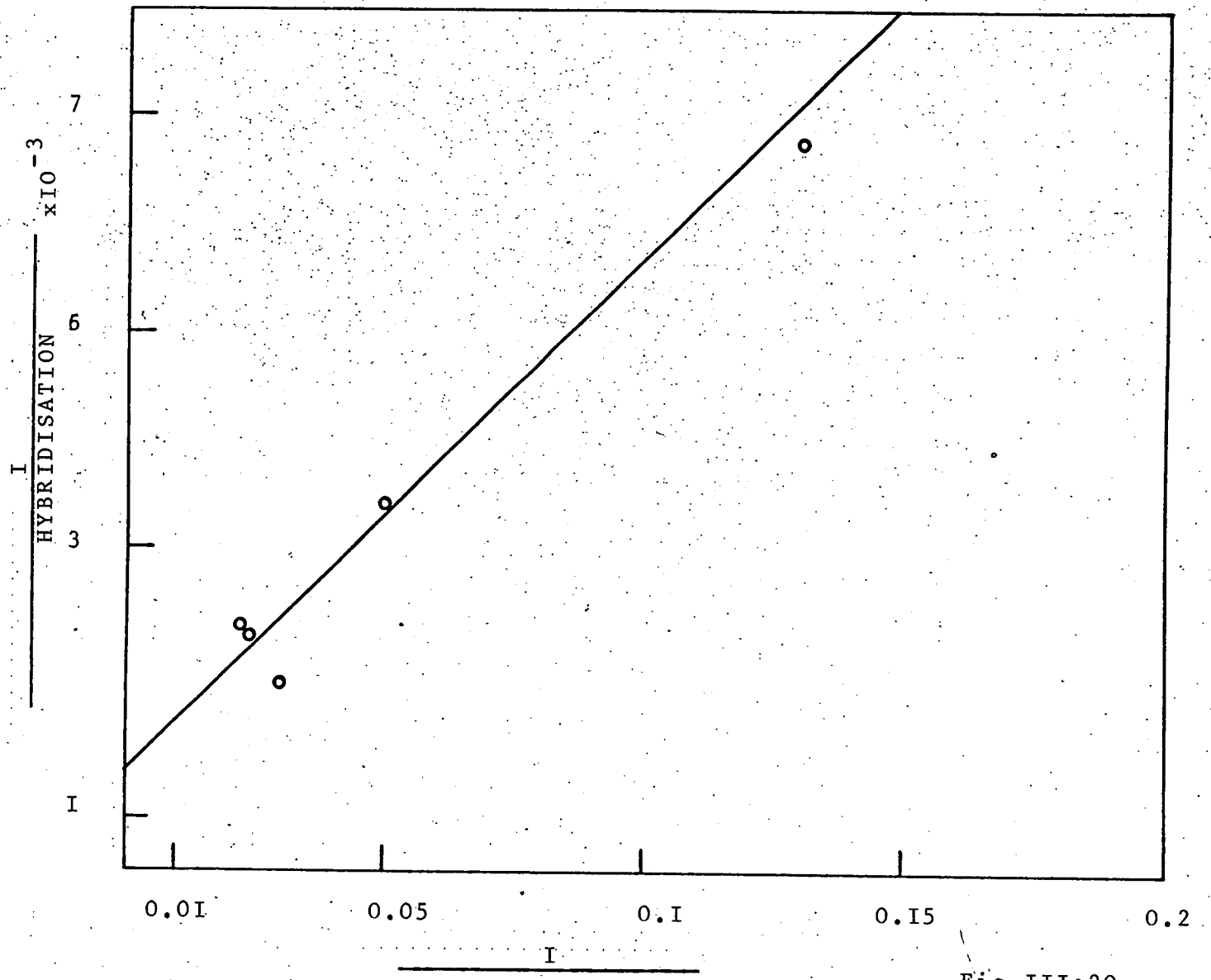

7
6
3
1
$\frac{1}{\text{HYBRIDISATION}} \times 10^{-3}$
0.01
0.05
0.1
0.15
0.2
$\frac{1}{\text{TIME (Hours)}}$

Fig.III:20.

Figure III:2I. Hybridisation of Adenovirus I2 cRNA synthesised off a denatured template to Adenovirus I2 DNA immobilised on filters. Hybridisation at T.OPT. (see Figure III:I9) in 6xSSC 30%FA with 5ng virus DNA and 2ug carrier M.luteus DNA added per filter.cRNA (S.A. I.7 xI0^{7} cpm/ug) was added at an original excess of 2:I cRNA to DNA and was heated at I00^{o}C for I5mins. prior to hybridisation.

Fig.III:2I.

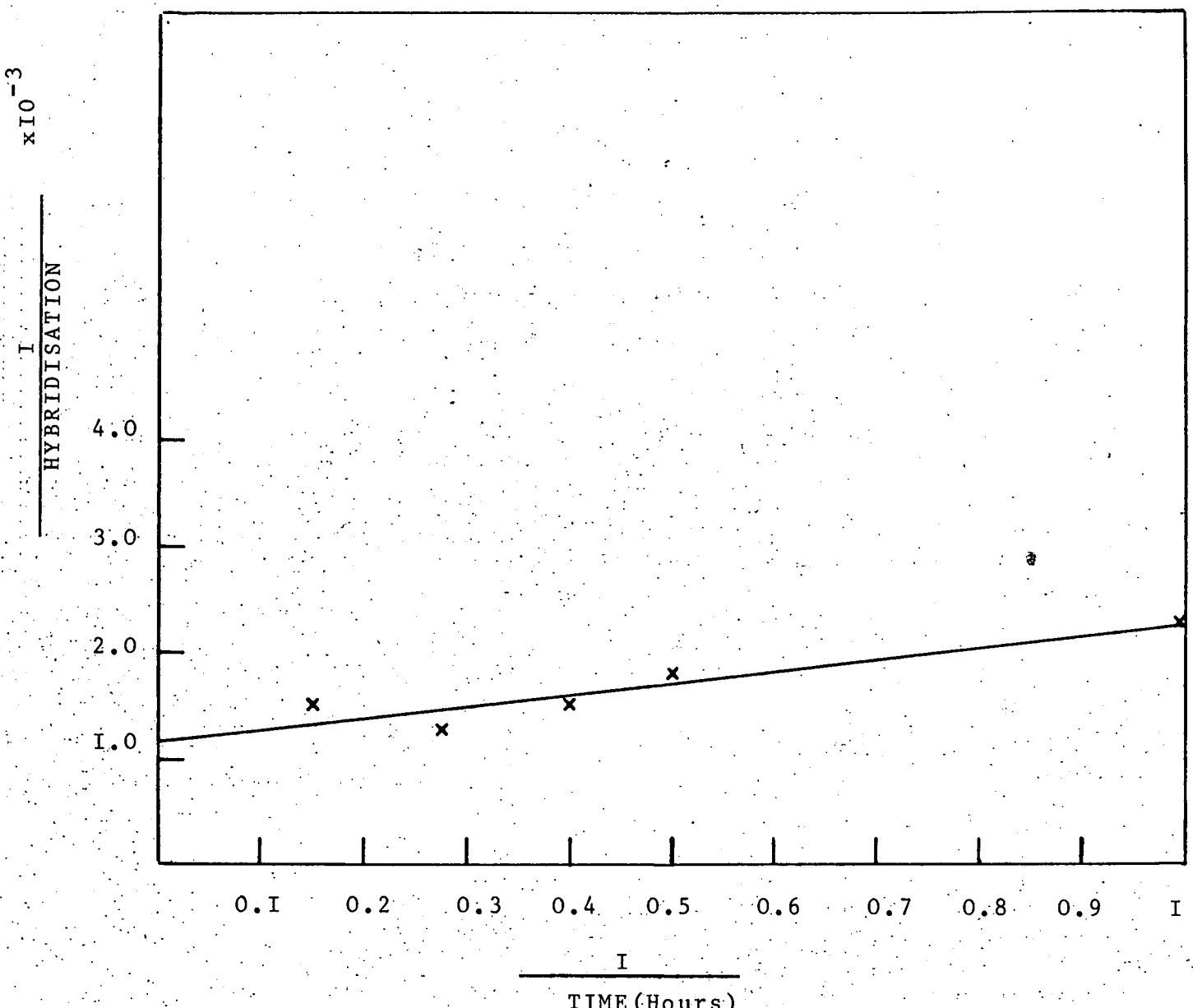

$\frac{I}{\text{HYBRIDISATION}} \times 10^{-3}$
4.0
3.0
2.0
1.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
$\frac{I}{\text{TIME (Hours)}}$

Table III:11

cRNA	T.OPT. (6xSSC 30% FA)	T.OPT. (1xSSC)	Complementary DNA (%)	$t\frac{1}{2}$(3ug/ml)	Approx. Kinetic Complexity (daltons x 10^{-6})
Adeno-2 denatured	53	62	n.d.	n.d.	-
Adeno-7 denatured	50	59	6-7	20-25 min.	1.3
Adeno-12 denatured	50	60	4	1 min.	0.06
Mouse satellite denatured	43	52	5	8-10 seconds	0.01

GENERAL CONCLUSIONS

There are a few important conclusions derived from the results of experiments described in this section.

First of all, cRNA excess experiments show that the majority of sequences in Adenovirus cRNAs appear to be transcribed from only a limited region of the Adenovirus genomes. The amount of DNA transcribed varies between different Adenovirus serotypes; Adenovirus 2 cRNA transcription possesses greatest selectivity; Adenovirus 12 has the least. Selectivity in transcription is an interpretation of the low saturation values and the unexpectedly low $t\frac{1}{2}$ values for individual hybridisation reactions (Table III:5). That the cRNA sequences detectable by cRNA excess hybridisation represent a majority of transcripts rather than sole transcripts is suggested by the slight increase in $Crt\frac{1}{2}$ under high cRNA excess conditions (Table III:7); by the fact that some of the cRNA sequences do hybridise to heterologous Adenovirus DNA serotypes under DNA excess hybridisation while they do not under cRNA excess hybridisation (Tables III:9; III:10), and also by the fact that cRNA sequences in very low concentration would theoretically fail to hybridise in the cRNA excess experiments (e.g. Bishop, 1972b). A second important point to emerge from the results presented here is the fact that the majority of cRNA sequences are probably AT-rich since the T.OPT. for hybridisation is always low (Table III:4) and the Tms for the cRNA-DNA hybrids are also in agreement with this (Table III:4).

Selective transcription of Adenovirus DNA by E. coli RNA polymerase has been noted by other workers (Green and Hodap, 1972; Dunn et al., 1973; Pettersson et al., 1974). In particular, these

last authors have shown that all the DNA sequences are represented in Adenovirus 2 cRNA but there is still preferential transcription, certain regions of the template being more active than others. By following the effect of adding unlabelled cRNA to the reassociation of labelled virus DNA fragments produced by E.COR 1 restriction enzyme, these workers showed that two Adenovirus 2 DNA fragments-D and F - were transcriptionally more active than the others. Both these fragments represent 7.11% and 4.70% of the Adenovirus 2 genome i.e. a total of 11.81% of the duplex. RNA transcribed from these regions would therefore represent about 5-6% of the genome since the product RNA is, in the main, asymmetric. This % complementarity is similar to the value obtained here (Table III:4) which has been derived from cRNA excess experiments. These two specific fragments, furthermore, are located at the AT-rich end of the Adenovirus 2 DNA molecule (Kimes and Green, 1970). Thus the cRNA to these regions will be UA-rich. In this respect this cRNA is additionally similar to the postulated UA-rich cRNA sequences described here for all three Adenoviruses. As mentioned, Pettersson et al. (1974) also suggest that all the Adenovirus 2 DNA sequences are represented in the cRNA transcripts. Part of their procedure incorporates hydroxyapatite fractionation which can lead to over-estimation of duplex or hybrid formation, particularly for Adenovirus DNAs (Tibbetts et al., 1973); so it is unclear if all the Adenovirus 2 DNA sequences really are represented in the cRNA. Although this remains to be precisely demonstrated, it is clear that a large proportion of the Adenovirus cRNAs are complementary to only certain regions of the Adenovirus genomes. The fact that there is selective transcription of

Adenovirus DNAs by the E. coli RNA polymerase means that experiments utilising Adenovirus cRNAs to detect virus DNA sequences in eukaryote cells have to be treated with caution. Most of the molecular hybridisation experiments such as cRNA excess and in situ hybridisation are directly influenced by the heterogeneity and concentration of the RNA species. Clearly, if most of the Adenovirus 12 cRNA, for example, only represents 20% of the Adenovirus 12 DNA template, then the chances of detecting Adenovirus 12 DNA sequences in eukaryote cells will be reduced. Another important point is that because the Adenovirus cRNA-DNA hybrids have relatively low Tms and T.OPTs (see Table III:4), to obtain maximum amounts of hybrid formation the incubation temperature has to be relatively low. For instance, hybridisation of Adenovirus 2 cRNA at 80°C in 2 x SSC (or its equivalent in a Formamide solution) would reduce the chances of detecting Adenovirus 2 DNA homology. This could not be predicted from the Tm of the virus DNA which is high GC (see Table III:1). A final point which needs to be mentioned is the fact that virus DNA sequences in certain transformed or tumour cells may only represent partial genomes (see Introduction). Therefore, the majority of Adenovirus cRNA sequences might not even be complementary to these partial genomes.

All this clearly demonstrates that before estimates or location of Adenovirus DNA sequences in transformed or tumour cells in particular can be made, the Adenovirus cRNAs and the Adenovirus cRNA-DNA hybrids have to be characterised.

SECTION IV

IN SITU HYBRIDISATION

The *in situ* hybridisation method (John *et al.*, 1969; Gall and Pardue, 1969) combines the specificity of molecular nucleic acid hybridisation with cytological discrimination leading to the detection and localisation of specific nucleic acid base-sequences within eukaryote cells (Jones, 1973; Hennig, 1973; Steffensen and Wimber, 1972).

Apart from chromosomal mapping of DNA sequences, one of its advantages over other conventional hybridisation methods is the fact that single cells can be studied. This can be an important consideration since some cells in a population might possess more, specific, DNA sequences than others: for example developing *Xenopus oocytes* have several times the amount of ribosomal genes than do follicle cells (Birnstiel *et al.*, 1970). Thereis also a restricted distribution of EBV genomes to the epithelial cells of certain human nasopharyngeal carcinomas (Wolf *et al.*, 1973; Klein *et al.*, 1974).

As regards virus-host cell interactions, the method has been utilised in several attempts to detect virus nucleic acids in permissive, semi-permissive, or non-permissive systems.

Replicating virus DNA has been detected *in situ* in SV40-infected monkey kidney cells (Geuskens and May, 1974); HRIK cells which are permissive for the replication of EBV (Pagano, 1974); and HEK cells infected with Adenovirus 12 (McDougall *et al.*, 1972; Dunn *et al.*, 1973). The applicationsof *in situ* hybridisation to semi-permissive systems are exemplified in the detection of Adenovirus 2 DNA replication in rat embryo cells (Gallimore, 1974), and the localisation of

replicating SV40 DNA in a small percentage of rabbit kidney cells transformed by this virus (Watkins, 1973). There have also been attempts to detect virus-specific DNA within virus transformed or tumour cells; for example certain SV40 transformed human cells and hamster tumour cells (Oda et al., 1972); and a cell line originating from an African Burkitt's lymphoma (Zur Hausen and Schulte-Holthausen, 1972). Shope papilloma virus DNA has been localised in rabbit skin (Orth et al., 1970), and for Adenoviruses there is the suggestion that virus DNA can be detected within the nuclei of transformed rat cells (Dunn et al., 1973) and rat tumours induced by Adenovirus 12 (Dunn et al., 1973; McDougall et al., 1972b).

Clearly, the method has been of great use in studying and clarifying the variety of virus-cell interactions which can occur.

For in situ hybridisation the principal parameters are the specific activity of the labelled nucleic acid "probe" and the amount of specific nucleic acid sequences in the cell to be analysed.

Briefly, the greater the specific activity, and the larger the amount of "target" sequences, the greater will be the chance of detecting them. Thus there is a greater chance of detecting virus DNA in infected cells than transformed or tumour cells since, in general, transformed or tumour cells contain lower amounts of virus DNA (see Introduction, Chapter 1).

There are two important facets of the in situ hybrdisation process which need to be considered in relation to its resolving power. The first is efficiency. Clearly, if the efficiency is low then this reduces the limit of detection. In conjunction with this is a second consideration of whether or not in situ reactions can be optimised.

Conventional hybridisation reactions can be optimised (section 111, this Chapter) with the direct result that their efficiency can sometimes be increased. Many of the features of in situ hybridisation appear to be similar to the RNA excess hybridisation method: for instance in both cases the denatured DNA is immobilised and RNA is incubated in solution around it.(also see Jones, 1973; Hennig, 1973). This suggests that in situ hybridisation reactions also can be optimised and their efficiency improved. This is particularly important, as in the present work, when the detectibility of trace amounts of nucleic acids in the cell is in question. Some of the work in this Thesis attempts to detect virus DNA sequences in transformed or tumour cells by in situ hybridisation (see following section). A knowledge of the efficiency of the process and its comparability with conventional RNA-DNA hybridisation is therefore desirable.

Experimental design

The effects of the fixation procedure and denaturation treatment have already been mentioned (see Materials and Methods, pg. 46).

Two types of experiment were designed to determine whether individual in situ hybridisation reactions could be optimised and to determine their efficiency. One type (B) monitors RNA-DNA hybrid formation (radioactivity) by autoradiography which is a normal feature of the in situ method; the other type (C) uses direct scintillation counting. Both types of experiment use satellite DNA as a test system because it is readily isolatable, easily detectable by both conventional and in situ hybridisation, and it has discrete chromosomal locations (see Walker, 1970 for example).

Satellite cRNAs were characterised and the conditions of satellite cRNA-DNA hybrid formation determined. In situ hybridisation reactions were then performed and the results compared with conventional hybridisation.

The reactions studied were mouse satellite cRNA-DNA and human satellite cRNA-DNA hybridisation.

The results show that individual reactions at the cytological level can be optimised and further, the optimal hybridisation conditions are similar to those found for conventional RNA excess hybridisation. Estimates of efficiency, carried out under optimal conditions, fall into the range of 4-5%.

A. CHARACTERISATION OF SATELLITE cRNAs

RESULTS & DISCUSSION

1. RNA transcribed from native duplex satellite DNA.

The E. coli RNA polymerase has a predilection for the H(heavy) strand of duplex mouse satellite DNA resulting in a 5-6 fold concentration of H strand transcripts over L(light) strand transcripts in the cRNA (Figure III:22).

Such hybridisation to both H and L strands of mouse satellite DNA could represent partial symmetric transcription of the satellite DNA, and this suggestion is supported by self-annealing experiments with mouse satellite cRNA which indicate 4-15% self-complementarity in the transcript (Table III:12).

The self-complementarities of the human satellite cRNAs are listed in Table III:12 and the values suggest a very limited amount of symmetric transcription of these satellite DNAs.

The sizes of the cRNAs were determined by sucrose gradient

Figure III:22. Hybridisation of mouse satellite cRNA to the complementary strands of mouse satellite separated by alkaline CsCl centrifugation. M. luteus and Xenopus DNAs are added as buoyant density markers. The DNA of each fraction was denatured, loaded onto millipore filters (Materials and Methods) and hybridised at the T.OPT. (see Figure III:24) in 6xSSC 30% FA, with a IO times excess of complementary satellite cRNA, for 2 reaction half-lives ($t_{\frac{1}{2}}$).

Fig.III:22.

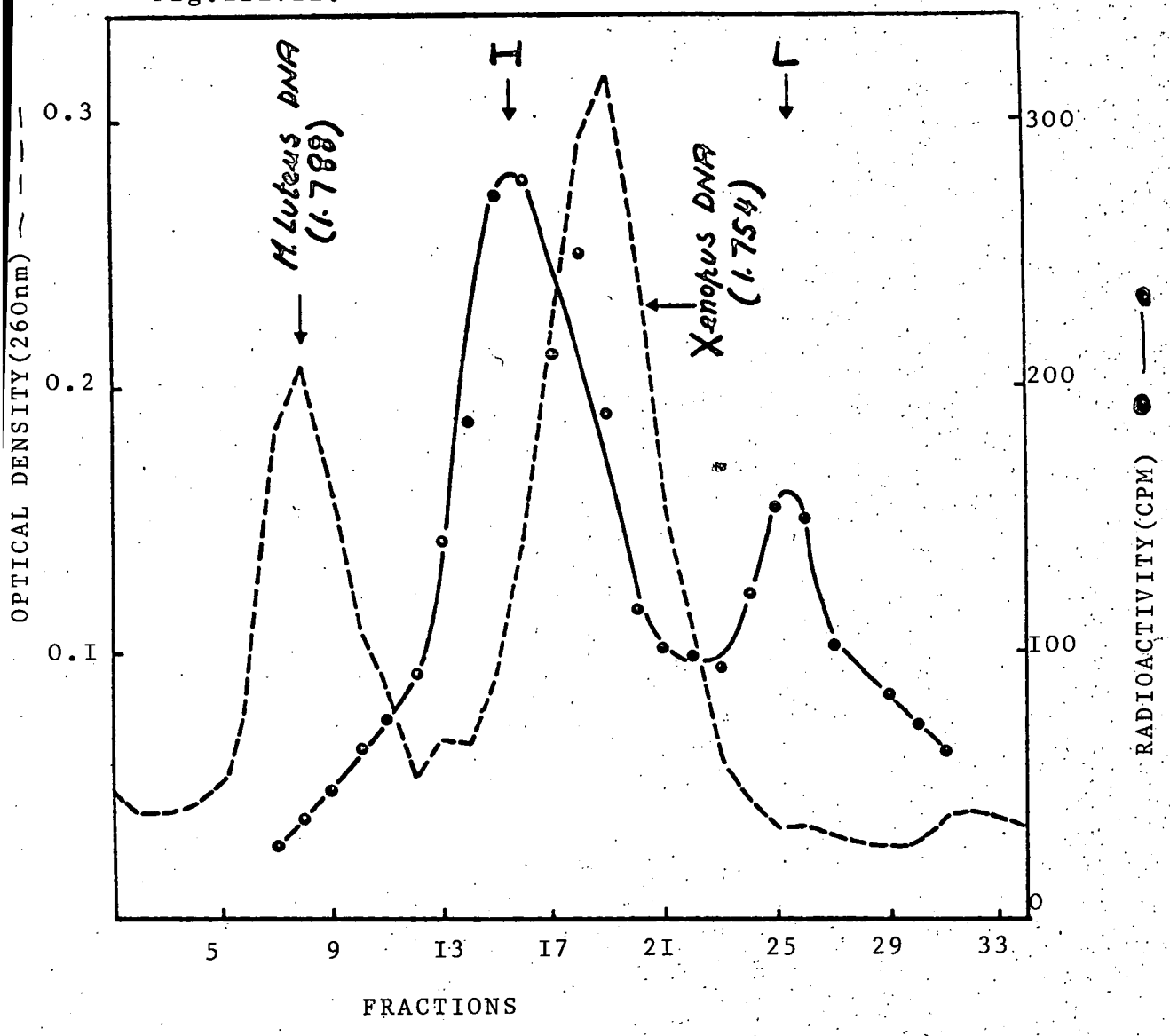

M.Lutens DNA (1.788)
H
L
Xenopus DNA (1.754)
OPTICAL DENSITY(260nm) ~ - - - -
0.3
0.2
0.1
300
200
100
0
RADIOACTIVITY(CPM) —●—
5
9
13
17
21
25
29
33
FRACTIONS

Figure III:23. Fractionation of mouse satellite cRNA(3×10^4 cpm) in a sucrose density gradient(5-40%).Spun at $15^{\circ}C$, 24,000 rpm for 18hours.RNA TCA precipitated.*E.coli* RNA added as a density marker.

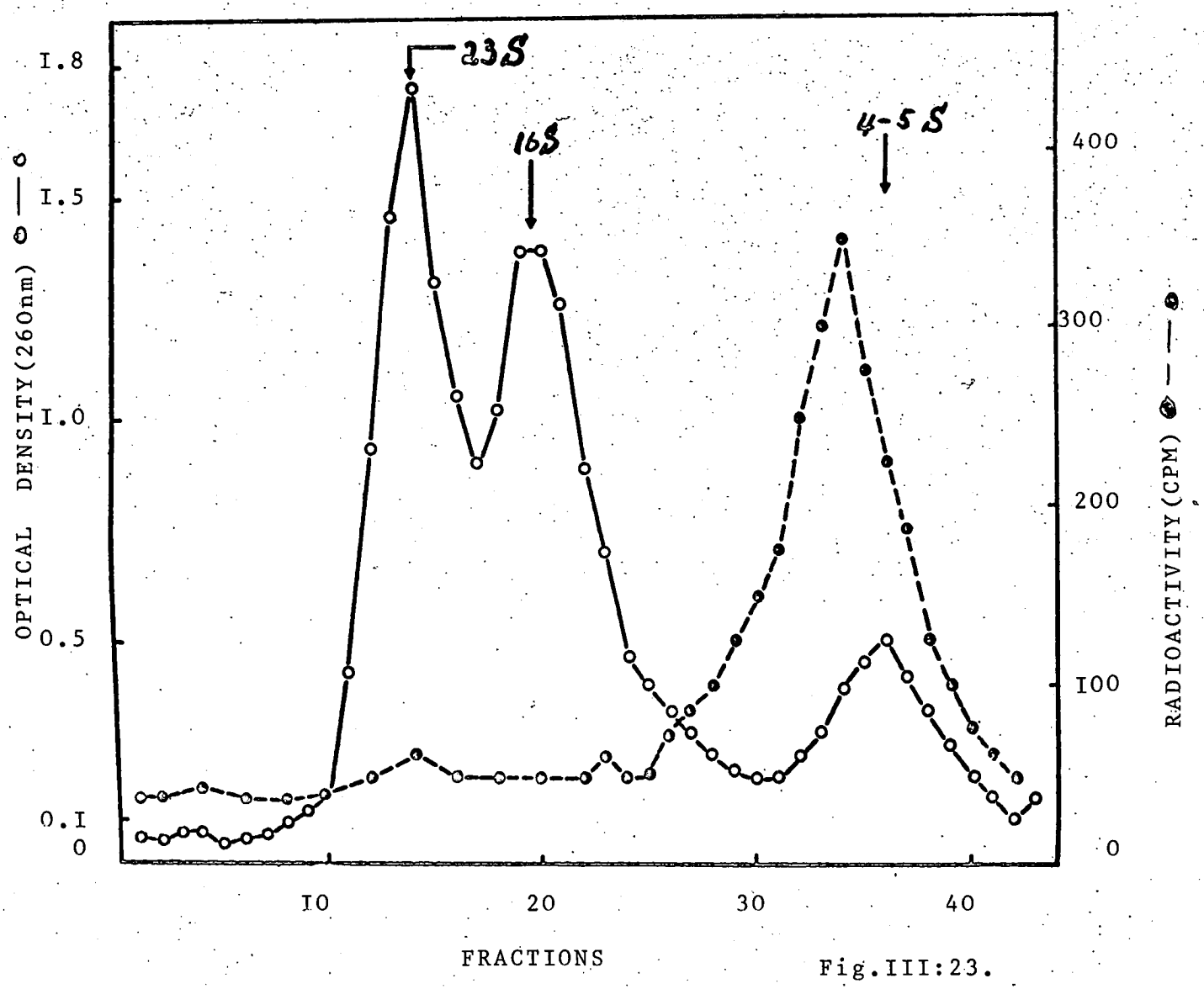

23S
16S
4-5S
1.8
1.5
1.0
0.5
0.1
0
OPTICAL DENSITY(260nm) o — o
400
300
200
100
0
RADIOACTIVITY(CPM) ● — ●
10
20
30
40
FRACTIONS

Fig.III:23.

centrifugation and were estimated to be between 5-6S, corresponding to molecules of 150-200 nucleotides (Figure III:23).

2. Optimal rate temperatures for hybridisation of satellite cRNAs

The temperature optimums (T.OPT.) for the formation of satellite cRNA-DNA hybrids are shown in Figure III:24. The T.OPTs for human satellite III and mouse satellite cRNAs were also determined in 1 x SSC and 3 x SSC (Table III:13).

Mouse satellite DNA has a Tm of 86°C in 1 x SSC (Bond et al., 1967) while human satellite DNAs I, II and III have Tms, in 1 x SSC of 80°C, 84°C and 85°C respectively (Corneo et al., 1968; Corneo et al., 1970; Corneo et al., 1971). These values are 30-40°C higher than the T.OPT. values for the individual satellite cRNA-DNA hybridisations, a temperature difference rather higher than previously reported for a variety of DNA-RNA hybrids (Birnstiel et al, 1972; Bishop, 1972).

3. Gradient Hybridisation

Mouse satellite cRNA was hybridised to mouse DNA fractionated on either a CsCl gradient (Figure III:25) or a Ag^{+}-Cs_2SO_4 gradient (Figure III:26) at the T.OPT. in appropriate hybridisation solution.

Human satellite III cRNA was hybridised to human DNA fractionated on a CsCl gradient at the T.OPT. for this reaction (Figure III: 27).

These gradient hybridisations show that the satellite cRNAs, for the most part, hybridise to DNA sequences with unique buoyant density.

cRNAs to total DNA, for instance total human DNA, hybridise to many sequences with a heterogeneous buoyant density (Figure III:28).

Figure III:24. Initial rate of satellite cRNA-DNA hybridisation as a function of temperature. cRNA was hybridised to filters containing total DNA.RNA concentration and time of incubation were chosen so that IO-30% of the complementary satellite DNA reacted.
a) 6xSSC 30%FA;b)3xSSC.
o-o Mouse satellite cRNA;□—□ human satellite III cRNA; ● — ● human satellite II cRNA; ■ — ■ human satellite I cRNA.

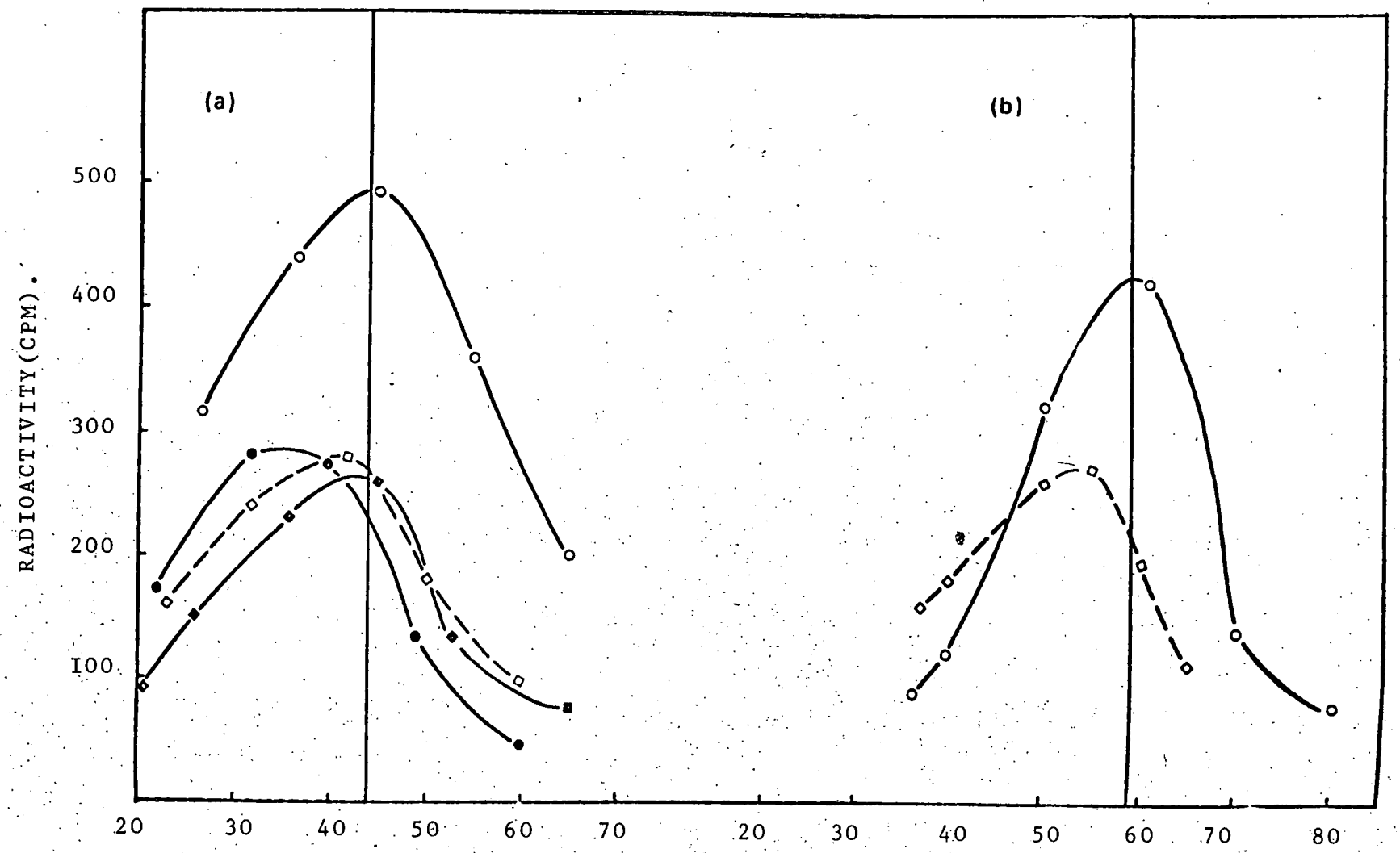

(a)
(b)
500
400
300
200
100
RADIOACTIVITY(CPM).
20
30
40
50
60
70
20
30
40
50
60
70
80
TEMPERATURE(°C)

Fig.III:24.

Figure III:25. Hybridisation of mouse satellite cRNA to total mouse DNA fractionated on a neutral CsCl gradient. Hybridisation as for Legend to Figure III:27. As well as hybridisation to DNA sequences with a buoyant density of mouse satellite DNA ($1.690 gm/cm^{-3}$) (see Table III:I), there is also some hybridisation to sequences which are present in the light part of the gradient. This may represent trailing of satellite sequences into this region of the gradient. Alternatively, some satellite sequences may not have been fully resolved into a satellite peak. These sequences may also be interspersed with the main band sequences. (also see Figure III:26).

Fig.III:25.

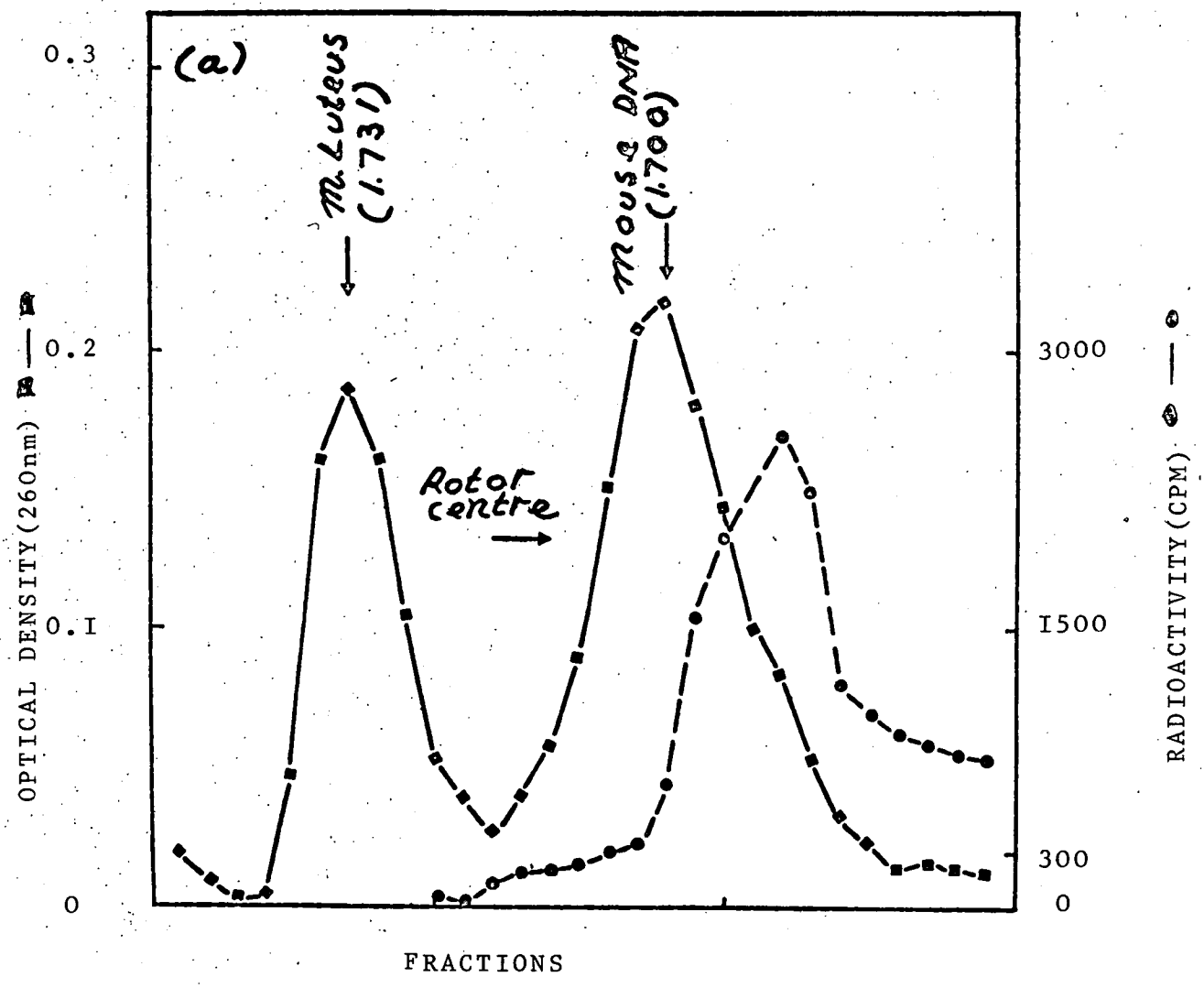

(a)
M.Lutens (1.731)
Mouse DNA (1.701)
Rotor centre
0.3
0.2
0.1
0
3000
1500
300
0
OPTICAL DENSITY(260nm)
RADIOACTIVITY(CPM)
FRACTIONS

Figure III:26. Hybridisation of mouse satellite cRNA(S.A. $I.4xI0^{7}$ cpm/ug) to total mouse DNA fractionated on a Ag^{+}-Cs_2SO_4 gradient. Gradients were made up as described in Materials and Methods (pg.49) at an R^{f} of 0.25, The DNA was centrifuged at 30Krpm for 72 hours at 25°C in the 8x60 MSE rotor, and the fractions were collected from the bottom of the tube. Aliquots were denatured, neutralised, and loaded onto membrane filters (The DNA sequences complementary to the cRNA were always very much less than the concentration of the cRNA sequences). 6xSSC 30%FA for $I0t_{\frac{1}{2}}$s at the T.OPT. After extesive washing in 2xSSC, followed by RNasing to remove unbound RNA, the filters were counted in Toluene-based scintillation fluid. Hybridisation is mainly to DNA sequences which on re-running on neutral CsCl band at a density of $I.690gm/cm^{-3}$ which is the buoyant density of mouse satellite DNA. The isolated satellite DNA in the Ag^{+}-Cs_2SO_4 gradient can also be observed by spectrophotometry at 260nm(arrow). Main band hybridisation can be accounted for by the presence of mouse satellite DNA sequences which probably have not been completely isolated by the technique of Ag^{+}-Cs_2SO_4 gradient centrifugation. Incomplete separation has been observed for the centrifugation of other satellite DNAs as well. (e.g. Prosser, I974).

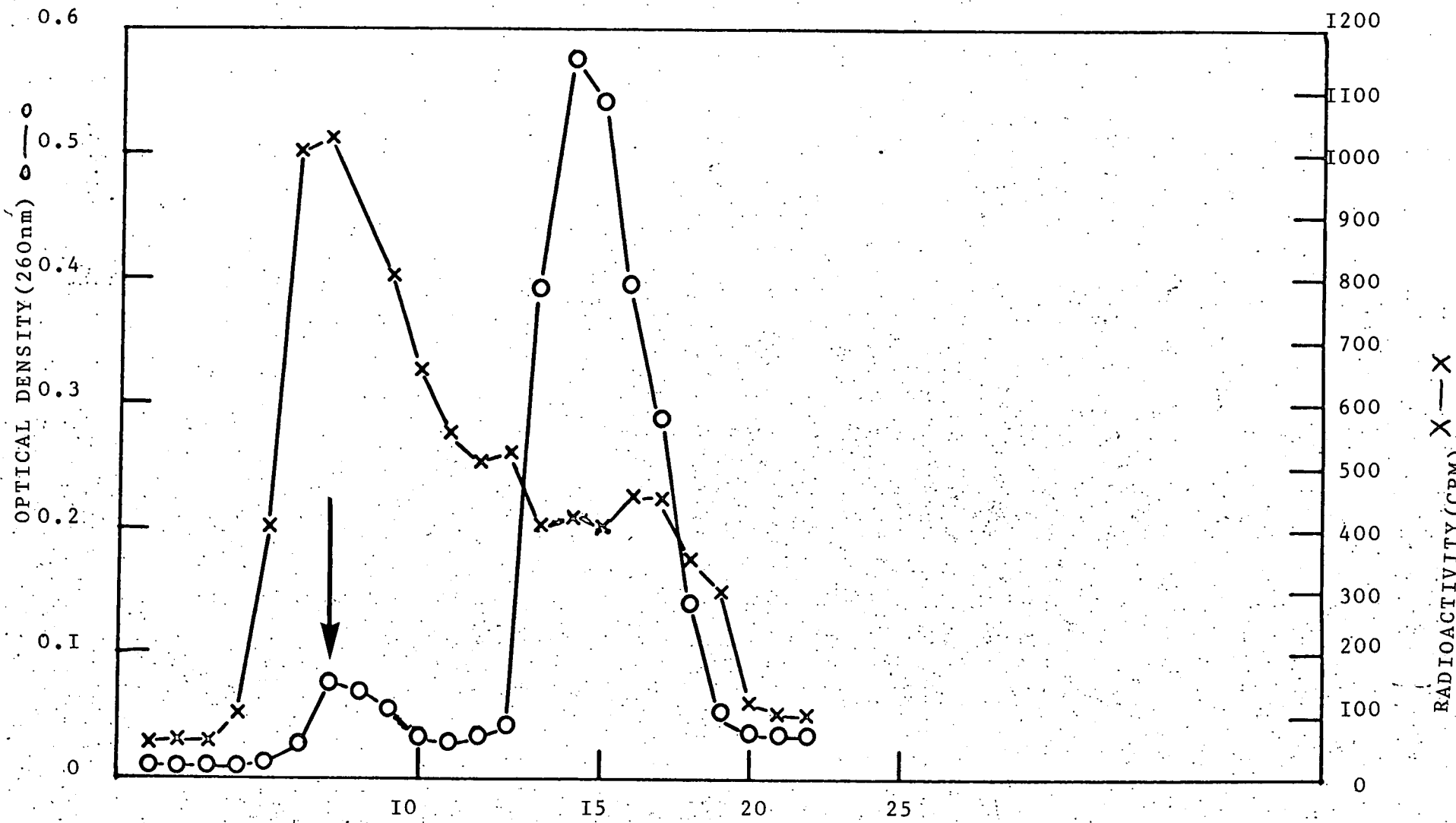

0.6
0.5
0.4
0.3
0.2
0.I
0
OPTICAL DENSITY(260nm) o—o
I200
II00
I000
900
800
700
600
500
400
300
200
I00
0
RADIOACTIVITY(CPM) X—X
I0
I5
20
25
CsCl FRACTIONS

Fig.III:26.

Figure III:27. Hybridisation of satellite cRNA to neutral CsCl gradient.DNA in O.IxSSC, together with M.luteus DNA as density marker, was added to 5.2gm CsCl and centrifuged in a MSE IOxIO rotor for 40hours at 25°C.Fractions were collected,their O.D.s(260nm) determined, and aliquots of each fraction denatured, loaded onto membrane filters and hybridised with human satellite III cRNA so that the concentration of the RNA was in a IO times excess of the complementary DNA on the filters.6xSSC 30%FA at the T.OPT. and continued for 2 reaction $t\frac{1}{2}$s.The peak of radioactivity corresponds to a buoyant density of I.696gm/cm^{-3}which is the buoyant density of human satellite III DNA in neutral CsCl(Corneo et al.,I973).

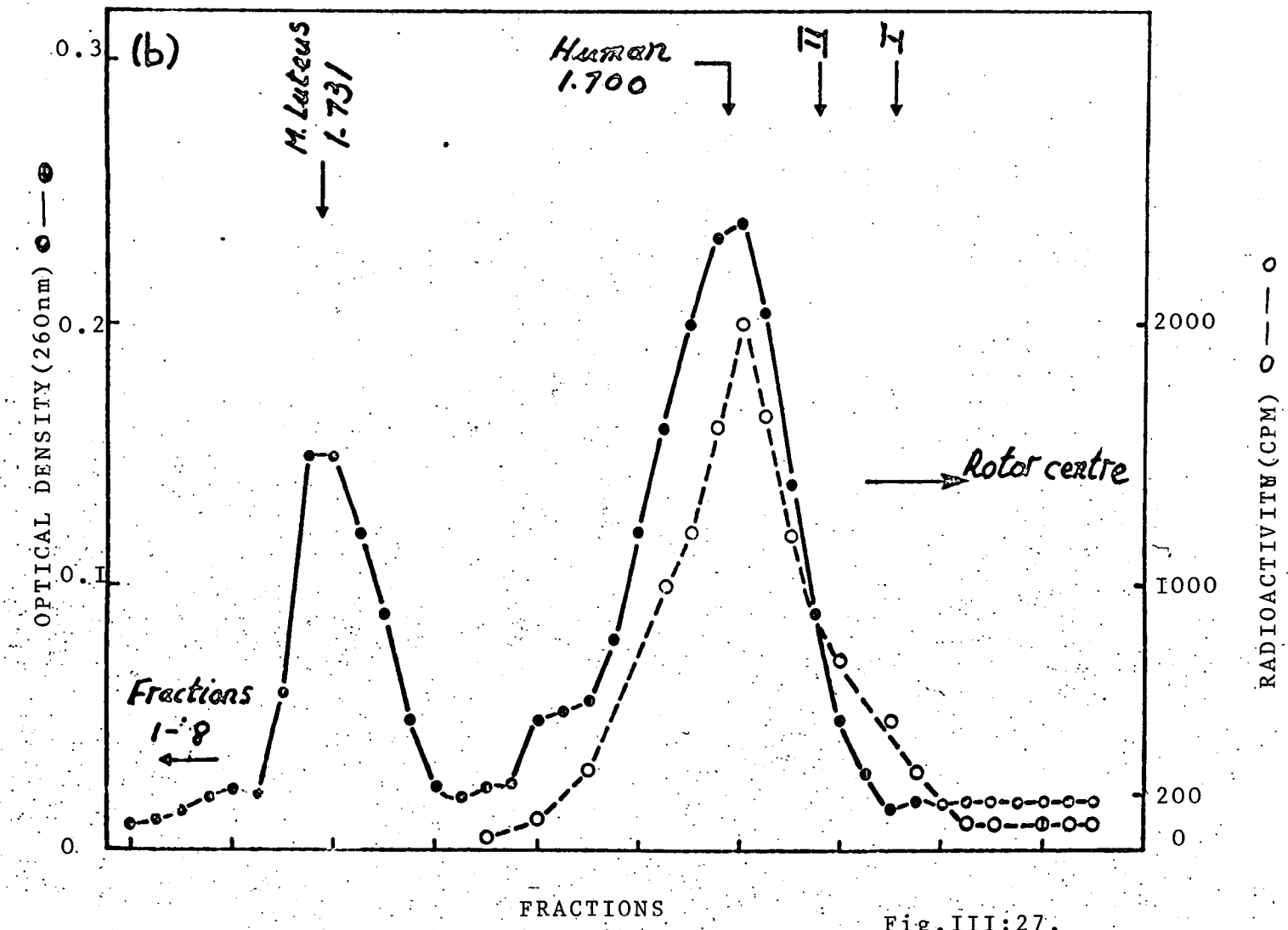

(b)
M. Luteus
1.731
Human
1.700
II
I
Rotor centre
Fractions
1-8
OPTICAL DENSITY (260nm) ●—●
RADIOACTIVITY (CPM) o—o
0.3
0.2
0.1
0
2000
1000
200
0
FRACTIONS

Fig.III:27.

Figure III:28. Hybridisation of total human DNA cRNA to total human DNA fractionated on a neutral CsCl gradient.Total Human DNA(0.00IM NaCl) was transcribed by the E.coli DNA-dependent RNA polymerase and the cRNA extracted and purified.The cRNA(S.A. 2.0x IO^7cpm/ug) was hybridised to total human DNA fractionated on the CsCl gradient as described in the Legend to Figure III:27.Hybridisation; to DNA sequences with heterogeneous buoyant density.

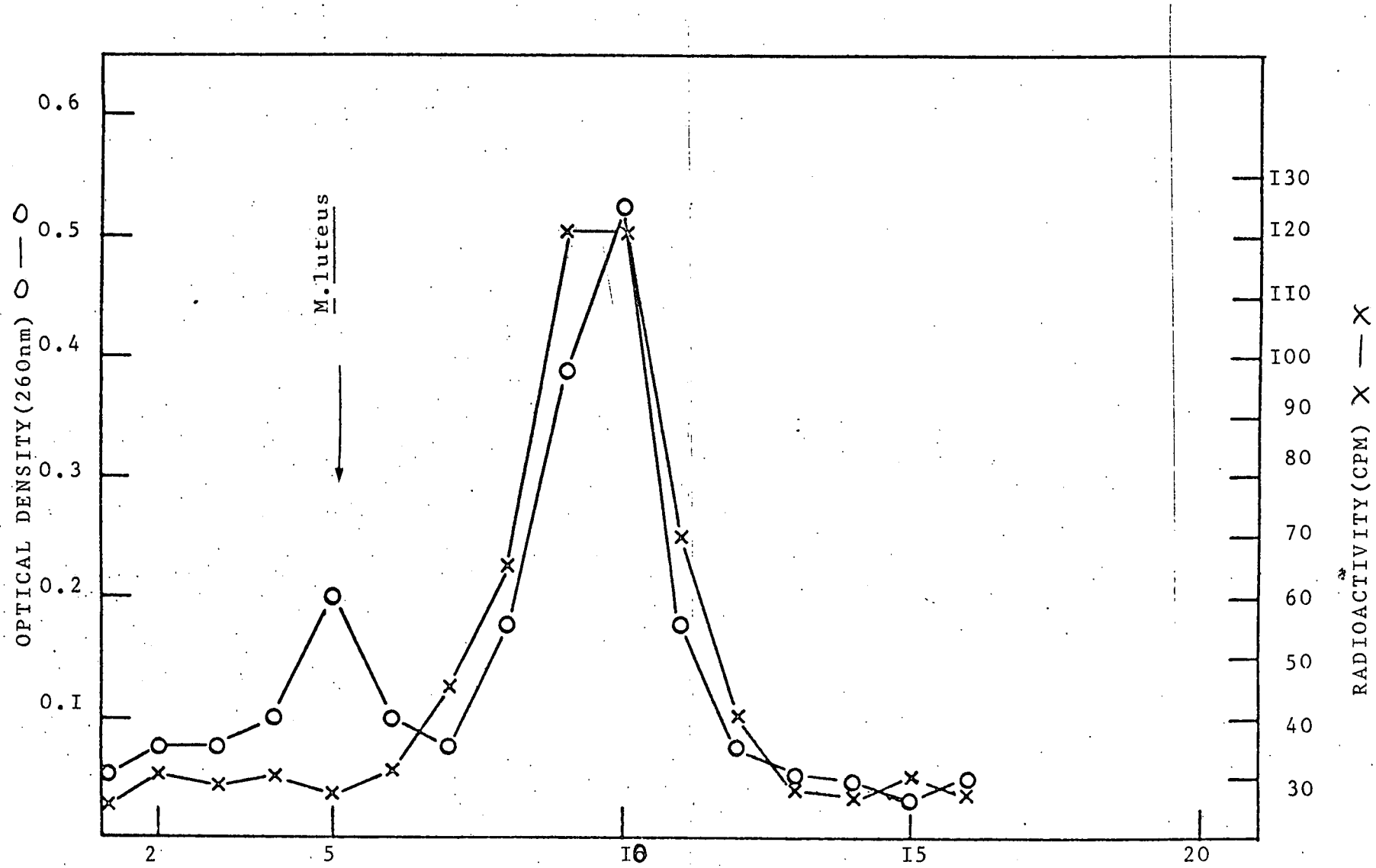

OPTICAL DENSITY(260nm) O — O
0.6
0.5
0.4
0.3
0.2
0.1
M.luteus
RADIOACTIVITY(CPM) X — X
I30
I20
II0
I00
90
80
70
60
50
40
30
2
5
I0
I5
20
CsCl FRACTIONS.

Fig.III:28.

Table III:12 Self-complementarity of satellite cRNAs

Satellite cRNA	Concentration (μg/ml)	%RNAse-resistance (c.p.m.-H^3) at several $T_{\frac{1}{2}}$s
Mouse	0.012	15
Mouse	0.014	7
Mouse	0.010	9
Mouse[a]	0.010	40
Human I	0.012	4
Human II	0.016	15
Human III	0.020	8

[a] Mouse cRNA transcribed from a denatured mouse satellite DNA template

4. Base-sequence complexity and rates of hybridisation of satellite cRNAs.

Figures III:29 and III:30 show double reciprocal linear plots for the hybridisation of satellite cRNA sequences. From the time taken to reach half-saturation, $Crt\frac{1}{2}$s were calculated (Table III:13).

Ø x 174 cRNA hybridises to its template DNA with a $Crt\frac{1}{2}$ of 15×10^{-3} moles/sec/l under RNA excess conditions at its T.OPT. ($45^{o}C$) in 6 x SSC 50% FA. (Birnstiel et al., 1972) and the DNA has analytical complexity of around 5500 bases. The $Crt\frac{1}{2}$ of mouse satellite cRNA is approximately 3×10^{-4} moles/sec/l and is therefore about 50 times less complex than the Ø x 174 cRNA. This infers that mouse satellite DNA has a kinetic complexity of around 100 base pairs, a value in agreement with complexities derived from renaturation studies (Waring and Britten, 1966; Sutton and McCallum, 1971; Hutton and Wetmur, 1973a). The kinetic complexities of the three human satellite DNAs are shown in Table III:13. The percentage of the mouse and human genomes complementary to the individual cRNAs can be calculated from the saturation values contained in the double reciprocal plots (Bishop, 1969; Birnstiel et al., 1972). While the value (Table III:13) for the percentage of the mouse genome complementary to the mouse satellite cRNA is in good agreement with previously published values (Kit, 1961), the percentages derived for each of the human satellite DNAs (Table III:13) are lower than have previously been suggested from densitometric measurements in the analytical ultracentrifuge (Corneo et al., 1973).

Figure III:30 demonstrates that even at low temperatures of incubation, where the rate of hybridisation is slower, full saturation is eventually achieved. As the temperature is increased so the rate of the reaction increases, although at supra-optimal temperatures the saturation value decreases as a consequence of greater RNA-DNA hybrid dissociation as the temperature approaches the Tm of the hybrid. For mouse satellite cRNA-DNA hybridisation, a reduction of 15°C from the T.OPT. leads to a 1.5 times decrease in reaction rate; and an increase of 15°C over the T.OPT. leads to a 1.75 increase in reaction rate but a 60% decrease in the saturation value.

5. Thermal stability of satellite cRNA-DNA hybrids

Figure III:31 shows the melting profiles of mouse satellite cRNA-DNA and the human satellite cRNA DNA hybrids originally formed at the T.OPT. The Tms of the hybrids are 12-18°C lower than the corresponding Tms for the native satellite DNAs (Table III:13), and are about 22°C higher than the T.OPT. values under similar criteria. The Tms of the mouse satellite and human satellite III cRNA-DNA hybrids formed also at 65°C are shown in Table III:13. They are 75-76°C and 72°C respectively.

B. In situ hybridisation of AT-rich satellite cRNAs

The optimal temperature of hybridisation, and the melting temperature of the AT-rich satellite cRNA-DNA hybrids studied here are 33-40°C and 12-18°C lower than the Tms of the native DNAs (Table III:13). This is a considerable temperature difference.

These particular satellite DNAs have already been assigned chromosomal sites (Jones, 1970; Pardue and Gall, 1970; Jones and Corneo, 1971; Jones, Prosser, Corneo and Ginelli, 1973; Jones et

Figure III:29. Double reciprocal plot for the reactions of human satellite IcRNA and human satellite III cRNA.Filters containing 0.0Iug or 0.05ug total human DNA were challenged with 0.02ug/ml satellite III or satellite I cRNA respectively,at the T.OPT.s in 6xSSC 30%FA for various times. S.A. of cRNAs was $I.4xIO^{7}$ cpm/ug.

Fig.III:29.

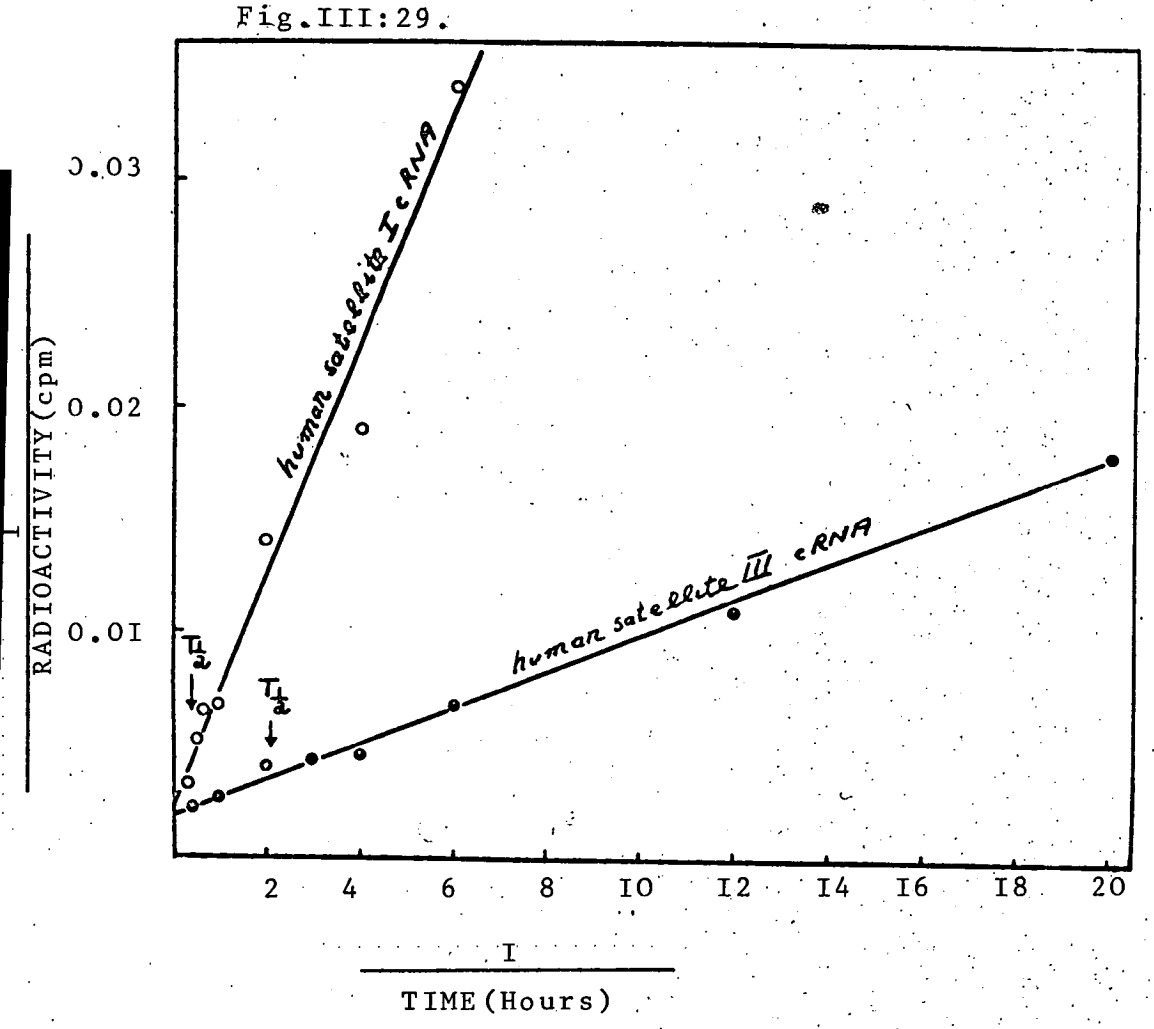

0.03
0.02
0.01
RADIOACTIVITY (cpm)
human satellite I cRNA
human satellite III cRNA
T1/2
T1/2
2
4
6
8
10
12
14
16
18
20
TIME (Hours)

Figure III:30. The effect of temperature on the rate of mouse satellite cRNA-DNA hybrid formation.cRNA excess. Filters containing 0.002ug total mouse DNA were hybridised in 6xSSC 30%FA until 80-90% of the reaction had been achieved.The cRNA(1.4×10^7 cpm/ug) was at a concentration of 0.018ug/ml. Each reaction is expressed as a double reciprocal plot and the difference in rates of the three linear plots can be expressed through the changes in the time for 50% saturation to be achieved.The reactions are essentially an experimental extrapolation of the optimal rate temperature curve shown in Figure III:24.

Fig.III:30.

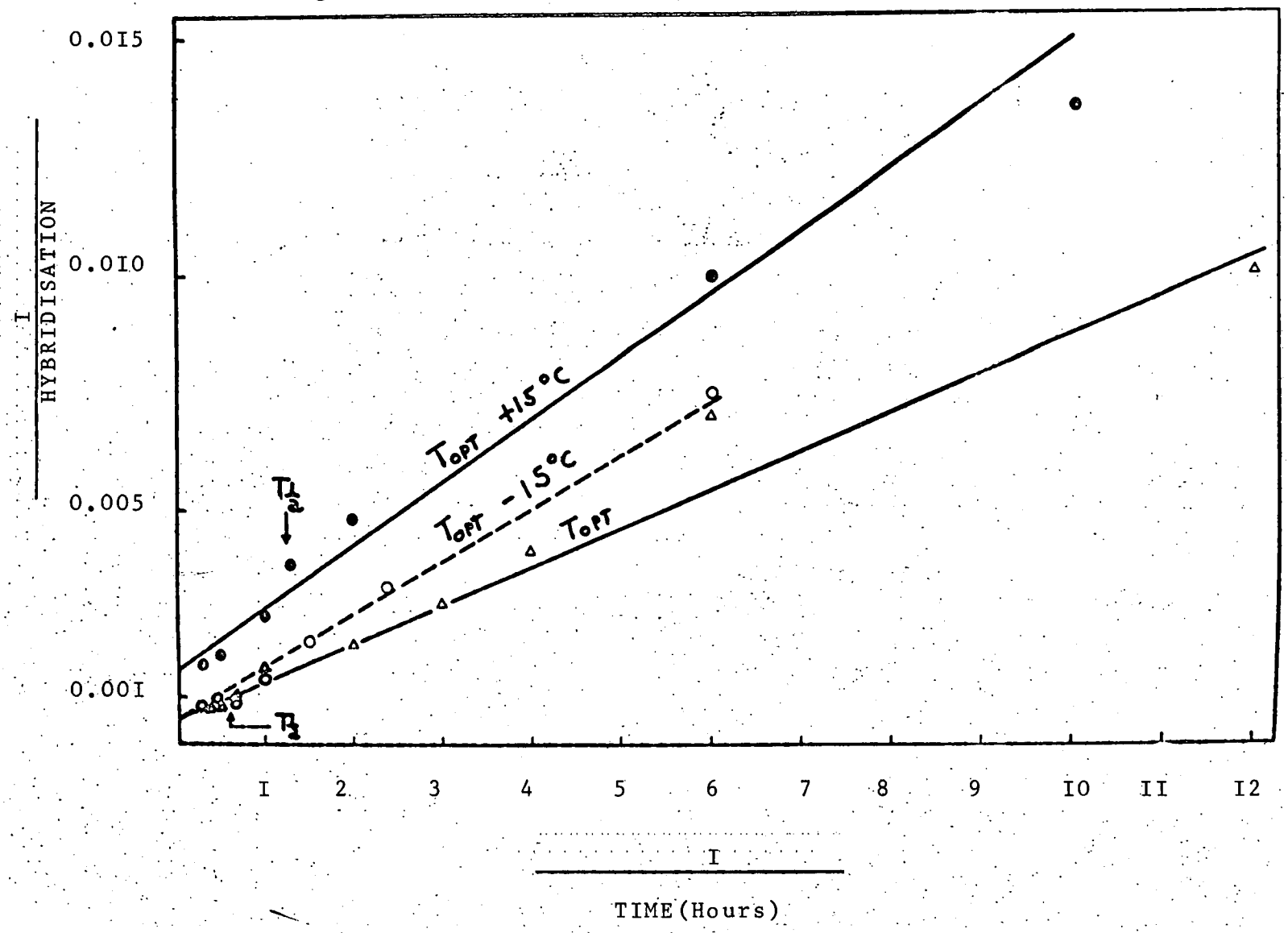

0.0I5
0.0I0
0.005
0.00I
1/HYBRIDISATION
T opt +15°C
T opt -15°C
T opt
T1/2
I
2
3
4
5
6
7
8
9
I0
II
I2
1/TIME(Hours)

Figure III:3I. Melting temperature profiles of cRNA-DNA hybrids. DNA was hybridised with saturating amounts of homologous satellite cRNA and the hybrids dissociated as described in Materials and Methods (pg. 42).
a) ■ - ■ Human III satellite cRNA-DNA hybrid;
□ - □ Human satellite II cRNA-DNA hybrid.
b) ● — ● Human I cRNAsatellite-DNA hybrid;
o-o mouse satellite cRNA-DNA hybrid.

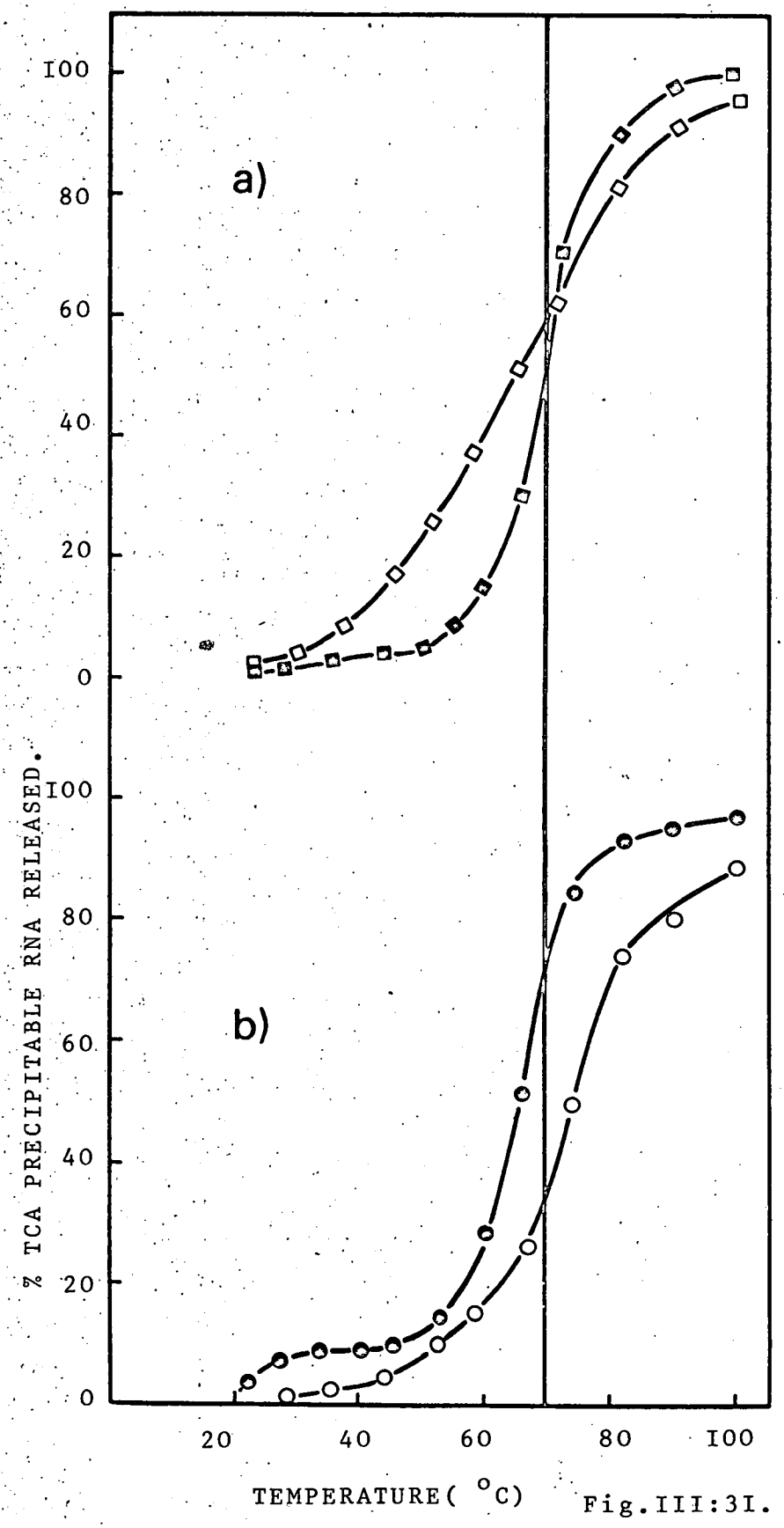

100
80
60
40
20
0
100
80
60
40
20
0
a)
b)
% TCA PRECIPITABLE RNA RELEASED.
20
40
60
80
100
TEMPERATURE(°C)

Fig.III:3I.

al., 1974) but apart from human satellite I the temperature of incubation chosen was generally 65°C which is already within the melting range of all these satellite cRNA-DNA hybrids (Figure III:31). This together with the findings that some of these satellite DNA sequences appear to have a restricted distribution in the karyotype suggested that reducing the temperature of incubation to without the melting range of the hybrids would lead to greater efficiency in the in situ hybridisation reaction. In turn, it could be investigated whether such an increase, if any, would reflect any basic similarities between in situ and conventional hybridisation.

RESULTS

Mouse satellite and human satellite III cRNAs were hybridised to mouse and human chromosomes respectively and hybrid formation monitored by autoradiography.

It can be seen from Figures III:32,III:33 and III:34 that the temperature of incubation is important in determining the amount of hybridisation that occurs in the karyotypes. At 75°C, for example, few chromosomes in either the mouse or the human are labelled (Figures III:33d and III:32c) whereas at 55°C several chromosomes are labelled (Figures III:33b and III:32b). In the human, and at this latter temperature, certain chromosomes from each group are consistently labelled near the centromeres: 1,7/8,9,10,11,13,14,15,16,17,18,19, 20,21,22 and in males a small heterochromatic chromosome most likely the Y (Figure III:32d). Mouse chromosomes, at 55°C with the exception of the y in males, are labelled at the centromeres. However there appears to be a variation in the amount of satellite DNA present on different chromosomes in this particular inbred strain

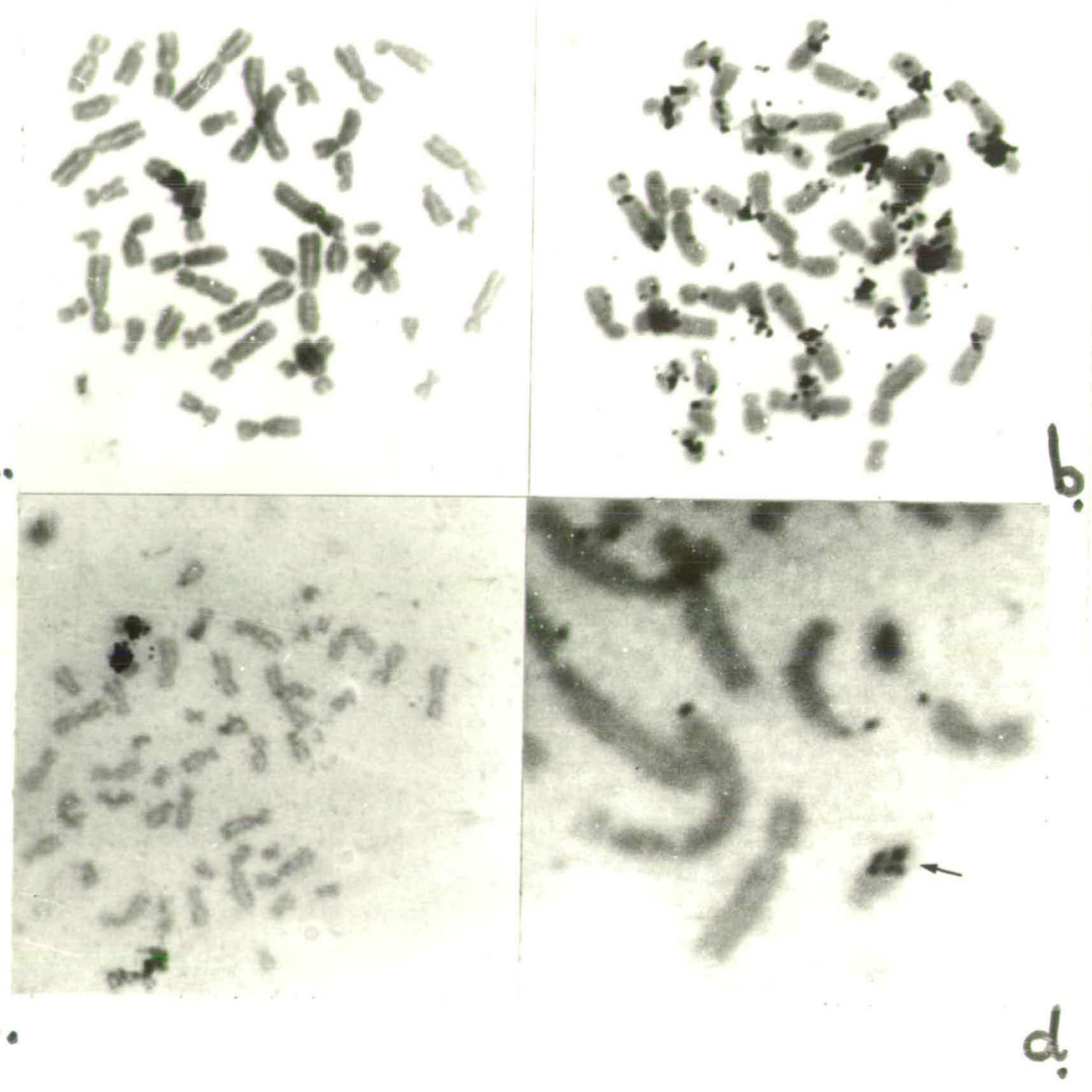
(JBT/Jd) which, like some other inbred mice (Dev. et al., 1974) has variations in the amount of C-band material on different chromosomes (Figure III:35). Chromosomes with minor amounts of C-band are only slightly labelled at 55°C (Figure III:33b) and are unlabelled at high temperature (Figure III:33d).

From Figures III:32, III:33 and III:34 it is also apparent that there is an optimal temperature of hybridisation for each of the satellite cRNA-DNA hybridisation reactions. For mouse satellite it is 50-60°C, for human satellite III it is also 50-60°C. By RNA excess hybridisation with DNA immobilised on membrane filters, the individual T.OPT.s for these reactions are 58°C and 53°C respectively (Table III:13). The T.OPT.s obtained both by conventional RNA excess hybridisation and in situ hybridisation are therefore in agreement. This suggests that both reactions share common kinetic characteristics.

DISCUSSION

Three important points arise from the results of experiments described here. First of all, there is an optimal rate temperature for the hybridisation of individual AT-rich satellite cRNA-DNA hybrids. Secondly, there is also an optimal temperature for the in situ hybridisation of these same satellite cRNA-DNA hybrids. Thirdly, these two optimums have similar values. This suggests that the in situ hybridisation reaction is markedly influenced by temperature (a point which may not have been fully appreciated in the past) in a way which reflects its basic underlying similarity to RNA excess hybridisation.

Figure III:32 and III:33. Mouse satellite and Human satellite III cRNAs were hybridised to mouse and human chromosomes respectively, by adding 2ul samples of the cRNA in 3xSSC(0.2ug/ml) to previously denatured (0.2N HCl) preparations.The reaction was carried out for 20mins..Autoradiographs were prepared as described.(Materials and Methods). Figure III:32 represents hybridisation of human satellite III cRNA to human chromosomes at a)20°C,b)55°C)75°C,d)70°C.Note the hybridisation of cRNA to the small chromosome, probably the Y(arrow).In Figure III:33,mouse satellite cRNA has been hybridised to mouse chromosomes at a)30°C,b)55°C,c)65°,d)75°C. The arrows identify chromosomes with differing amounts of satellite DNA, a finding most likely correlated with the amounts of C band material(see Figure III:35 and discussion).


a.
b.
c.
d.

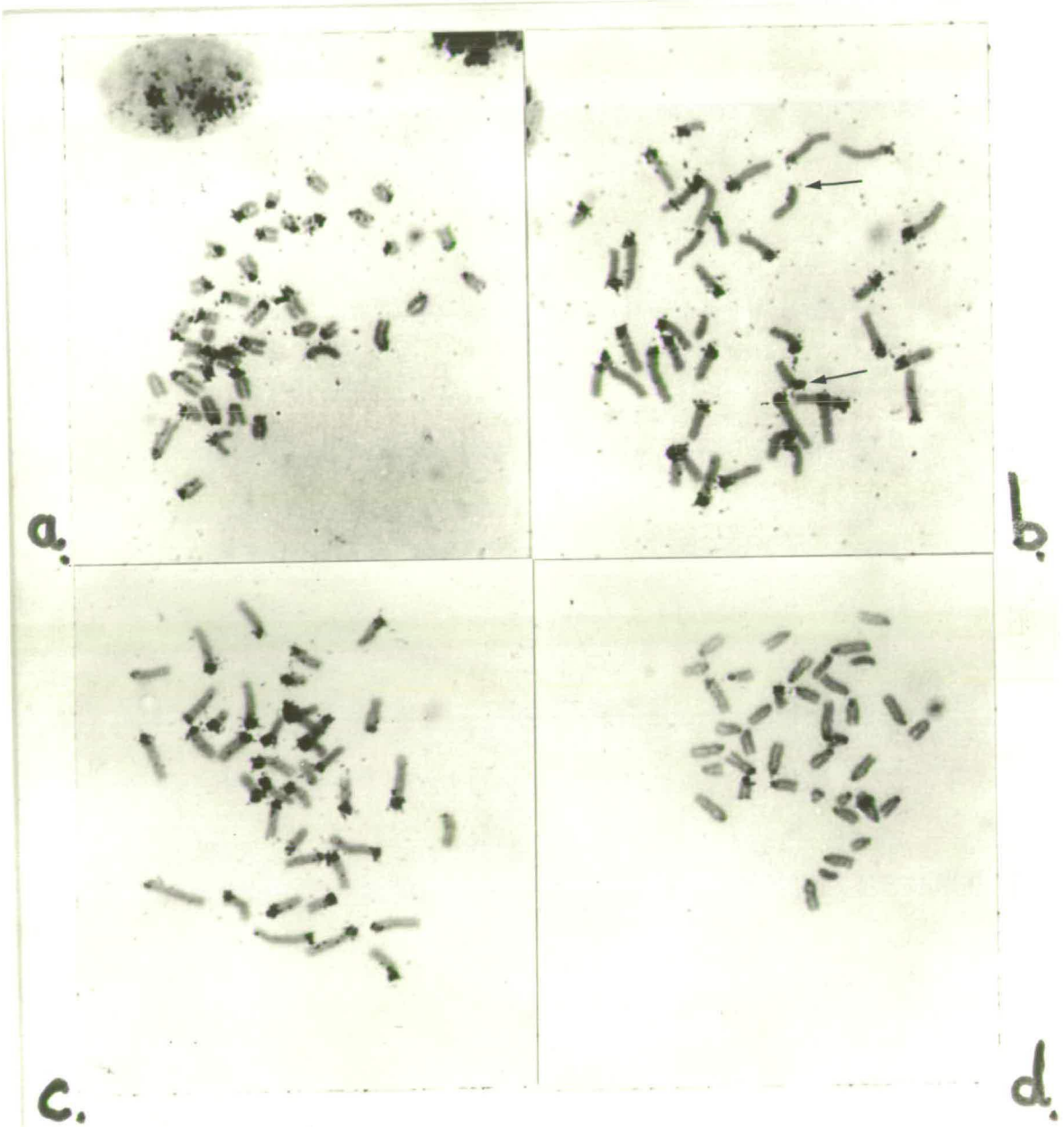

a.
b.
c.
d.

Figure III:34. The average number of chromosomes labelled after hybridisation with human satellite III and mouse satellite cRNAs,at different temperatures of incubation. Preparations were denatured with 0.2 N HCl. cRNA(in 3xSSC) was hybridised to the chromosomal DNA sequences for 20mins.,and autoradioagraphs were exposed for IOdays. A total of IOO metaphase spreads were examined at each temperature of hybridisation.

Fig.III:34.

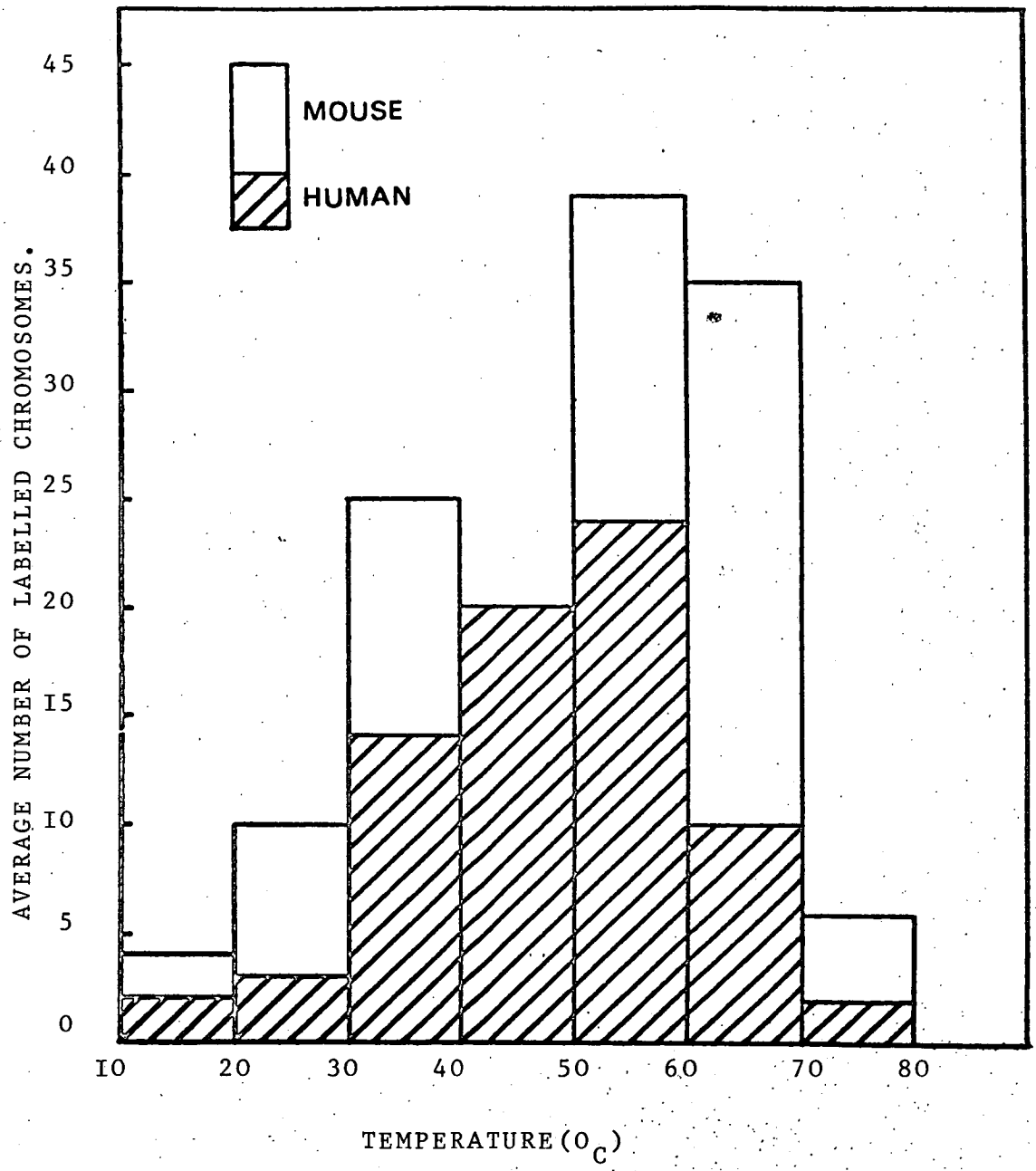

MOUSE
HUMAN
AVERAGE NUMBER OF LABELLED CHROMOSOMES.
0
5
10
15
20
25
30
35
40
45
10
20
30
40
50
60
70
80
TEMPERATURE (0C)

Figure III:35. Female chromosomes were denatured with 0.07N NAOH for 3 mins. and incubated in 2xSSC for Ihour at 65°C. The amount of C band material is variable between chromosomes, in particular two homologues possessing very much less than the others.

Table III:13

Satellite cRNA	T.OPT.$^{hybrid(H)}$ (1xSSC)	(3xSSC)	(6xSSC 30% FA)	Tm^{Hybrid} (1xSSC) T.OPT.	65°C	$Tm^{DNA(D)}$ (1xSSC)	Tm^{H}-T.OPT.^{H} (1xSSC)	Tm^{D}-T.OPT.^{H} (1xSSC)	$Tm^{D}-Tm^{H}$ (1xSSC)	$T\frac{1}{2}$(secs)* (3µg/ml)	$Crt\frac{1}{2}$ M/sec/l	Complexity nucleotides	% Genome
Mouse	52	58	43	74	76	86	22	34	12	24-38	$3x10^{-4}$	100	$11(10^{a}-12)$
Human I	47	-	38	66		80	19	33	14	50-60	$6x10^{-4}$	200	$0.175(0^{b}.5)$
Human II	44	-	35	66		84	22	40	18	26-36	$4x10^{-4}$	120	$0.7(2^{b})$
Human III	48	53	40	70	72	85	22	37	15	10-14	$1.2x10^{-4}$	40	$0.85(1^{b}.5)$

* 6xSSC 30% Formamide

a Waring and Britten (1966)

b Corneo et al. (1973).

In detail, the individual T.OPTs are low for several possible reasons. First, the satellite DNAs themselves have low Tms (Bond et al., 1967; Corneo et al., 1973). Second, cRNAs to AT-rich satellite DNAs will contain substantial regions of Uridine or Adenine residues which can impart low thermal stability to nucleic acid helices. Thirdly, the size of the RNA can be expected to have an effect; and finally, because satellites are composed of similar but not identical sequences, mismatching of these sequences upon reassociation or hybridisation will occur. These explanations gain support from the fact that individual Tms are low also (Figure III:31; Table III:13). The degree of these effects can be approximately determined from these Tms. Mouse satellite cRNA, for example, is mainly transcribed from the H strand of the DNA (Figure III:22) which is rich in Thymidine clusters (Southern, 1970) and will therefore be mostly composed of Adenine residues. The mixed ribose-deoxyribose homopolymer pair, rA-dT, melts 4-5^{o}C lower than dA-dT (Chamberlin, 1965). The length of the cRNA, which in this case is around 150-200 bases (Figure III:23), can have an important inverse effect on the Tm of a hybrid (Birnstiel et al., 1972) as well as the Tm of a DNA-DNA duplex (Thomas and Dancis, 1973). This, together with the fact that the minimum stable length of polyrA-polydT or polyU-polyA appears to be relatively long (Walker, 1969) suggests a 4-5^{o}C contribution to a reduction in Tm with an RNA length of approximately 150-200 base-pairs. Additional reduction in Tm comes from base-sequence mismatching. Thus the mouse satellite cRNA-DNA hybrid will contain around 2-5% mismatch and the human satellite III cRNA-DNA hybrid will contain around 3% more mismatch. These values

are close to mismatch estimates of 5% for reassociated mouse satellite DNA (Sutton and McCallum, 1971) and 7% for reassociated human satellite III DNA (Corneo et al., 1973). There may be rather more mismatch for the other human satellite cRNA-DNA hybrids since they have extended melting profiles as well as low Tms (Figure III:31).

The fact that the T.OPTs and the Tms for the formation and dissociation of AT-rich satellite cRNA-DNA hybrids are low means the rate of hybridisation is faster at relatively low temperature and second, the amount of tolerance of hybrid formation at the T.OPT. is higher than at more elevated temperature: i.e. the saturation value is higher. These two points are demonstrated in Figures III:34 and III:30 respectively.

That these two conclusions are important for the chromosomal mapping of these AT-rich satellite DNAs by in situ hybridisation is demonstrated by the results shown in Figures III:32, III:33 and III:34. Specifically, there is a temperature optimum at which more chromosomes are labelled than at any other temperature and this temperature optimum corresponds to the T.OPT. obtained by RNA excess experiments. A decrease in the amount of label at elevated temperatures, for example 75°C (see Figures 32c, 33d) is likely to be due to reduced saturation value: chromosomes with minor amounts of satellite DNA are unlabelled at high temperatures but are labelled at the T.OPT.: and chromosomes with large amounts of satellite DNA are labelled at all temperatures of incubation. For example, human chromosome 9 is known to contain a large amount of satellite DNA (Jones and Corneo, 1971; Jones, Prosser, Corneo and Ginelli, 1973) and both homologues of this chromosome are labelled at all temperatures

of incubation (Figure III:32) and even where 90% of the hybrid has melted (Figure III:31). Mouse satellite DNA exists as a 10% fraction (Waring and Britten, 1966; Kit, 1961) whereas human satellite 111 represents a much smaller proportion of the genome (Corneo et al., 1973; also see Table III:13). Thus hybridising mouse satellite cRNA even at temperatures where approximately 25% of the potential hybrid formed at the T.OPT. will not have formed (Figure III:31) most of the mouse chromosomes are still labelled (Figure III:33c). However, at temperatures where approximately 70% of the potential hybrid will not form the centromeric regions of several specific chromosomes are unlabelled (Figure III:33d). These have visibly less C band material (Figure III:35). A decrease in the amount of label at temperatures lower than the T.OPT. (Figures III:32, III:33 and III:34) may well reflect the fact that at these temperatures saturation is not achieved. The satellite cRNAs studied here hybridise with $Crt\frac{1}{2}s$ of 3×10^{-4} moles/sec/l (mouse) and 1.2×10^{-4} moles/sec/l (human 111) so that the 20 min. incubation time in the in situ reaction (see Materials and Methods) would normally represent approximately 10-20 reaction half-lives in 3 x SSC. Under these RNA excess conditions the reaction at low temperatures of incubation, for example 15°C below the T.OPT., would still have approached saturation (Figure III:30).Since temperatures such as these result in less numbers of labelled chromosomes, this suggests that the in situ hybridisation reaction may well be slower than that determined by RNA excess hybridisation.

Alternatively, the in situ hybridisation reaction may suffer from a depletion of RNA, for example not sufficient RNA excess (Birnstiel et al., 1972; Young and Paul, 1973) which has the effect of reducing

both the reaction rate and saturation value; or depletion of DNA sites, for example chromosomal DNA reassociation (Alonso *et al.*, 1974). This latter consideration is unlikely however in view of the findings of Kurnit (1974) who has shown that highly-repetitive chromosomal DNA sequences do not appear to renature during the C band procedure. Because *in situ* hybridisation and RNA excess hybridisation appear to share the characteristic of a common temperature optimum, experiments designed with this in mind may well increase the resolution of the technique. For example, many studies utilising *in situ* hybridisation have used incubation temperatures around 65°C as originally described by Jones (1970) and Pardue and Gall (1970). This temperature, however, is approximately 10°C above the T.OPT. for the formation of human satellite III cRNA-DNA hybrids and as such can be expected to lead to decreased saturation value for this reaction. Since Jones, Prosser, Corneo and Ginelli (1973) also used 65°C to determine that the location of human satellite lll DNA sequences is limited to a few chromosomes, their results can be considered a minimum estimate. The possibility of cross-hybridisation between human satellite lll cRNA and other satellite DNAs (e.g. Melli *et al.*, 1975) seems unlikely since highly labelled chromosomes at the T.OPT. which are not labelled at higher temperatures, do not correspond to those sites assigned to human satellite ll or l DNAs (Jones and Corneo, 1972; Jones *et al.*, 1974).

For other RNA species being used in the *in situ* hybridisation reaction, increased tolerance of hybrid formation might be particularly important since hybridising at the T.OPT. should increase the chances of detecting those chromosomal DNA sequences complementary to the RNA. The complex formation between polyU and chromosomal polydA

is a case in point (Shenkin and Burdon, 1974; Jones, Bishop and Brito-da-Cunha, 1973).

For the detection of virus DNA in eukaryote cells the above findings have similar implications. Thus hybridising Adenovirus cRNAs to cells at temperatures which are inside the thermal dissociation range of the potential hybrids will reduce the chances of detecting the virus DNA sequences. There is some evidence that this may have been done by other workers in some cases.

Clearly the fact that *in situ* hybridisation appears to behave as conventional hybridisation with regard to some basic parameters means, in effect, that the results of conventional hybridisation experiments in general can be applied to the designing of *in situ* hybridisation experiments in detail.

C. IN SITU HYBRIDISATION TO WHOLE CELLS FIXED ONTO COVERSLIPS

Mouse satellite cRNA was hybridised to mouse cells immobilised by fixation onto coverslips (Materials and Methods, pg. 56). cRNA-DNA hybrids were monitored by direct scintillation counting.

RESULTS

1. The effect of denaturation, temperature of incubation and other aspects of the normal *in situ* hybridisation procedure were investigated by observing their effect on ^{3}H-Thymidine labelled cells.

Figure III:36 shows that NaOH treatment (Gall and Pardue, 1969) removes major amounts of radioactivity from the coverslips whereas HCl treatment (MacGregor and Kezer, 1971) does not. Most of the loss with the former denaturant is due to whole cells being removed as well as DNA. HCl was routinely used. Figure III:37 shows that washing in

Figure III:36. Effect of denaturants on ^{3}H-Thymidine labelled cells fixed onto coverslips. o-o 0.07N NaOH; x-x 0.2 N HCl. Any concentration of NaOH did,in fact,remove DNA and cells.

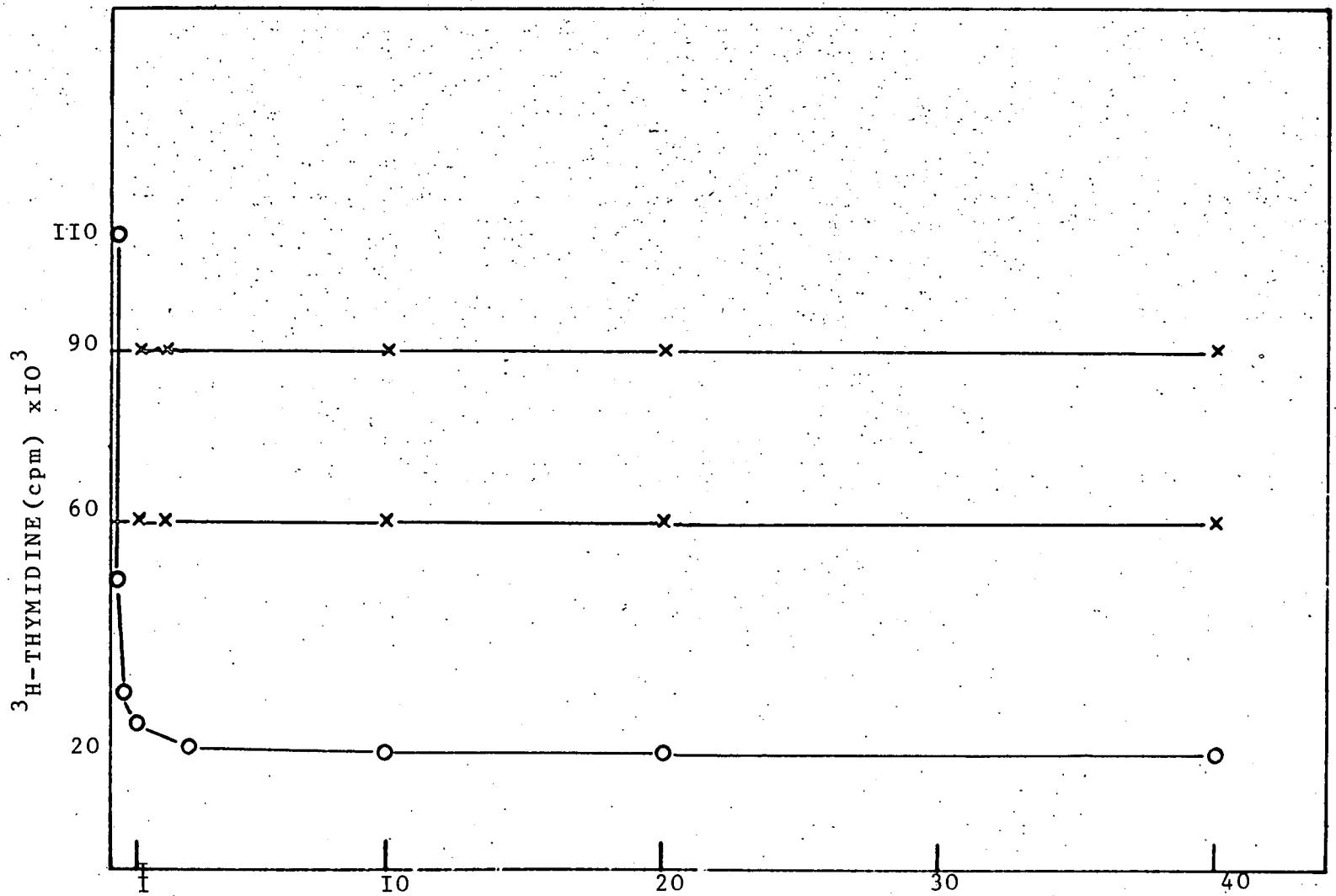


Fig.III:36.

Figure III:37. Effect of 2xSSC(25°C) washing and RNasing(25°C,IOug/ml in 2xSSC) on ^{3}H-Thymidine labelled cells fixed onto coverslips.Longer times still did not remove substantial amounts of radioactivity. o-o Rnase;x-x 2xSSC

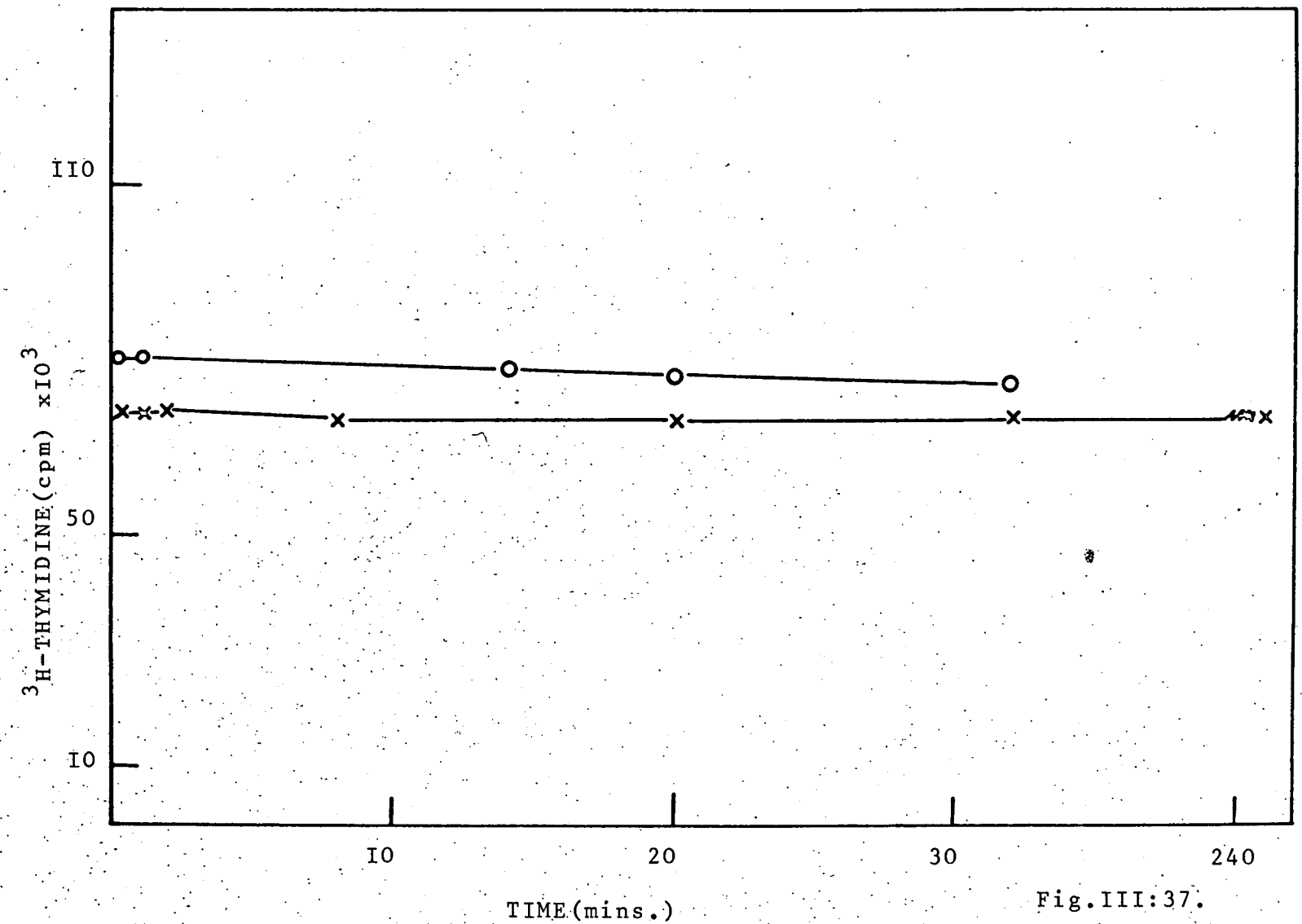


Fig.III:37.

Figure III:38. Effect of temperature on ^{3}H-Thymidine labelled cells fixed onto coverslips.Coverslips were heated in 2xSSC at different temperatures for 4-24 hours incubation.The time of incubation was not important compared to the influence of temperature.

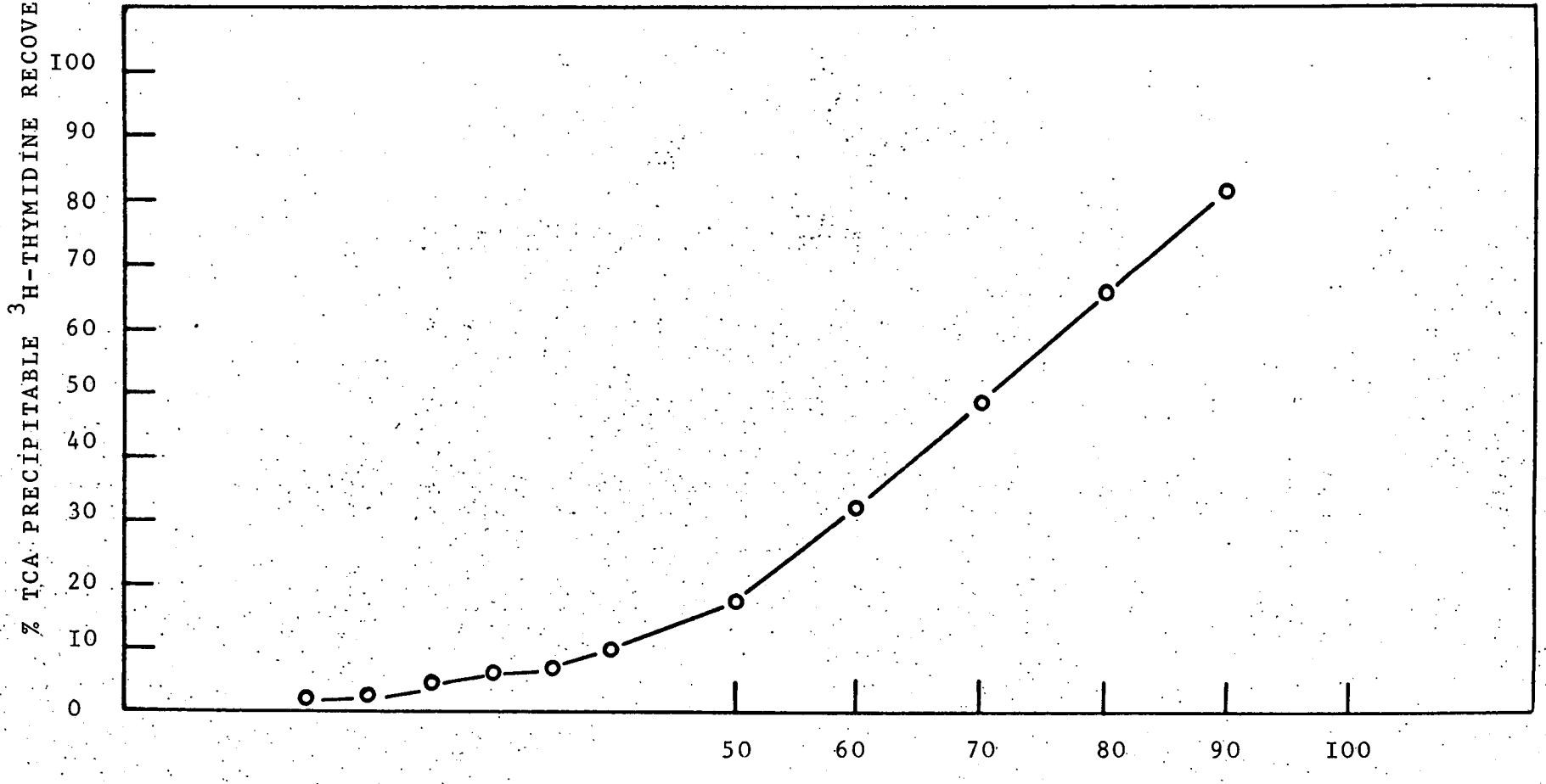

% TCA PRECIPITABLE ^{3}H-THYMIDINE RECOVERED.
100
90
80
70
60
50
40
30
20
10
0
50
60
70
80
90
100
TEMPERATURE (^{o}C).

Fig.III:38.

2 x SSC or RNAsing (20μg/ml) does not remove appreciable amounts of radioactivity. Figure III:38 demonstrates that temperature, however, has a marked effect on the retention of ^{3}H-Thymidine on the coverslip: the higher the temperature, the greater the loss. In SSC solutions, the optimum rate temperature of hybridisation of mouse satellite cRNA-DNA hybrid formation is quite high (Table III:13). Formamide, however, reduces the effect of temperature on the ^{3}H-labelled cells (Figure III:39), as well as reducing the optimal rate temperature of mouse cRNA-DNA hybrid formation (Table III:13). Formamide was therefore used in all the subsequent experiments on the kinetics of in situ hybridisation with whole cells fixed onto coverslips.

2. Mouse satellite cRNA-DNA hybrid formation.

Experiments discussed above have shown the T.OPT. to be important in in situ hybridisation reactions. The T.OPT. for mouse satellite cRNA-DNA hybrid formation in 6 x SSC 30% FA is 43°C (Table III:13). In 3 x SSC 50% FA the T.OPT. will be approximately 25°C. Hybridisation reactions were therefore carried out at 25°C in 3 x SSC 50% FA.

Figure III:40 shows that mouse cRNA hybridises specifically to mouse cells since it hybridises to mouse cells but not to human cells; mouse cells, however, do not hybridise with human satellite III cRNA. Hybridisation increases with time until it reaches a plateau. The mouse satellite cRNA-DNA hybrid melts in situ with a Tm of 37°C in 1 x SSC 30% FA (Figure III:39). Allowing for the effect of FA (0.7°C for 1% FA; McConaughy et al., 1969) this extrapolates to 72°C in 1 x SSC. The Tm of mouse satellite cRNA-DNA hybrid, formed on membrane filters in conditions of cRNA excess, is 74°C (Table III:13). There is therefore good agreement between these two values. A minimal amount of ^{3}H-Thymidine is lost from the coverslips under the same temperature

Figure III:39. Melting of DNA-DNA or cRNA-DNA hybrids from cells on coverslips.Cells, either labelled with ^{3}H-Thymidine or hybridised with mouse satellite cRNA at 25^{o}C in 3xSSC 50%FA,were exposed to temperature increments in a IxSSC 30%FA solution and the radioactivity released measured by TCA precipitation and counting in Toluene-based scintillation fluid.The Tm at which 50% of the radioactivity was released corresponds to 54^{o}C(^{3}H_Thymidine) or 37^{o}C(^{3}H-cRNA). x-x ^{3}H_Thymidine; o-o ^{3}H-cRNA.

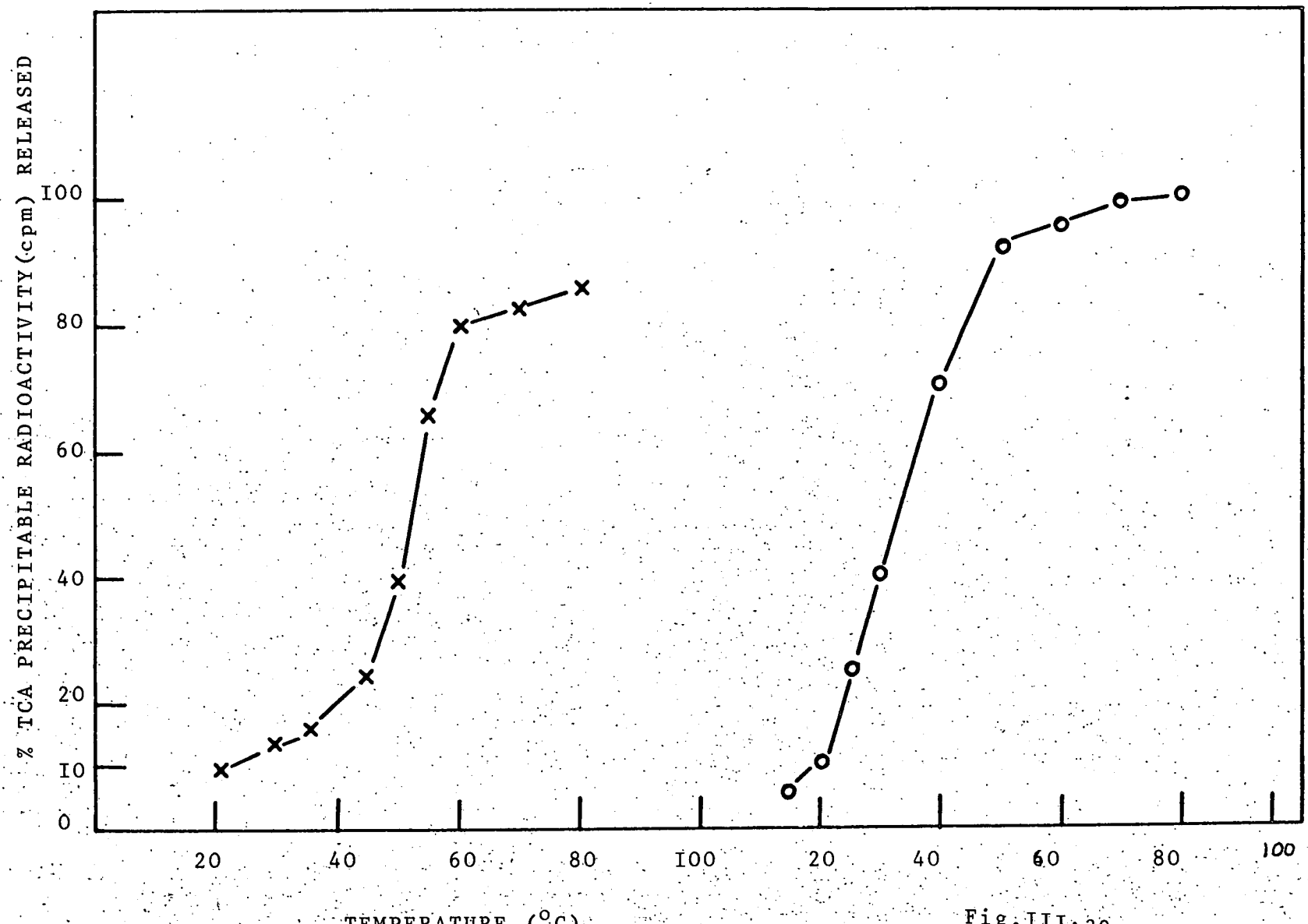

% TCA PRECIPITABLE RADIOACTIVITY (cpm) RELEASED
100
80
60
40
20
10
0
20
40
60
80
100
20
40
60
80
100
TEMPERATURE (°C)

Fig. III: 39.

Figure III:40. Hybridisation of satellite cRNA to cells fixed onto coverslips. Satellite cRNA (S.A. 1.4×10^{7} cpm/ug; 3×10^{-2} ug) in 3xSSC 50% FA was hybridised to cells (5×10^{4}/coverslip) fixed in 3:1 methanol:acetic acid. Temperature of incubation: 25°C. Radioactivity was monitored by direct scintillation counting i.e. counting the slips after post-*in situ* treatment (see Materials amd Methods, pg. 51). in Toluene -based scintillation fluid.
x-x Mouse satellite cRNA hybridised to mouse embryo cells; o-o Human satellite III cRNA hybridised to mouse embryo cells; O-O mouse satellite cRNA hybridised to human Hela cells.

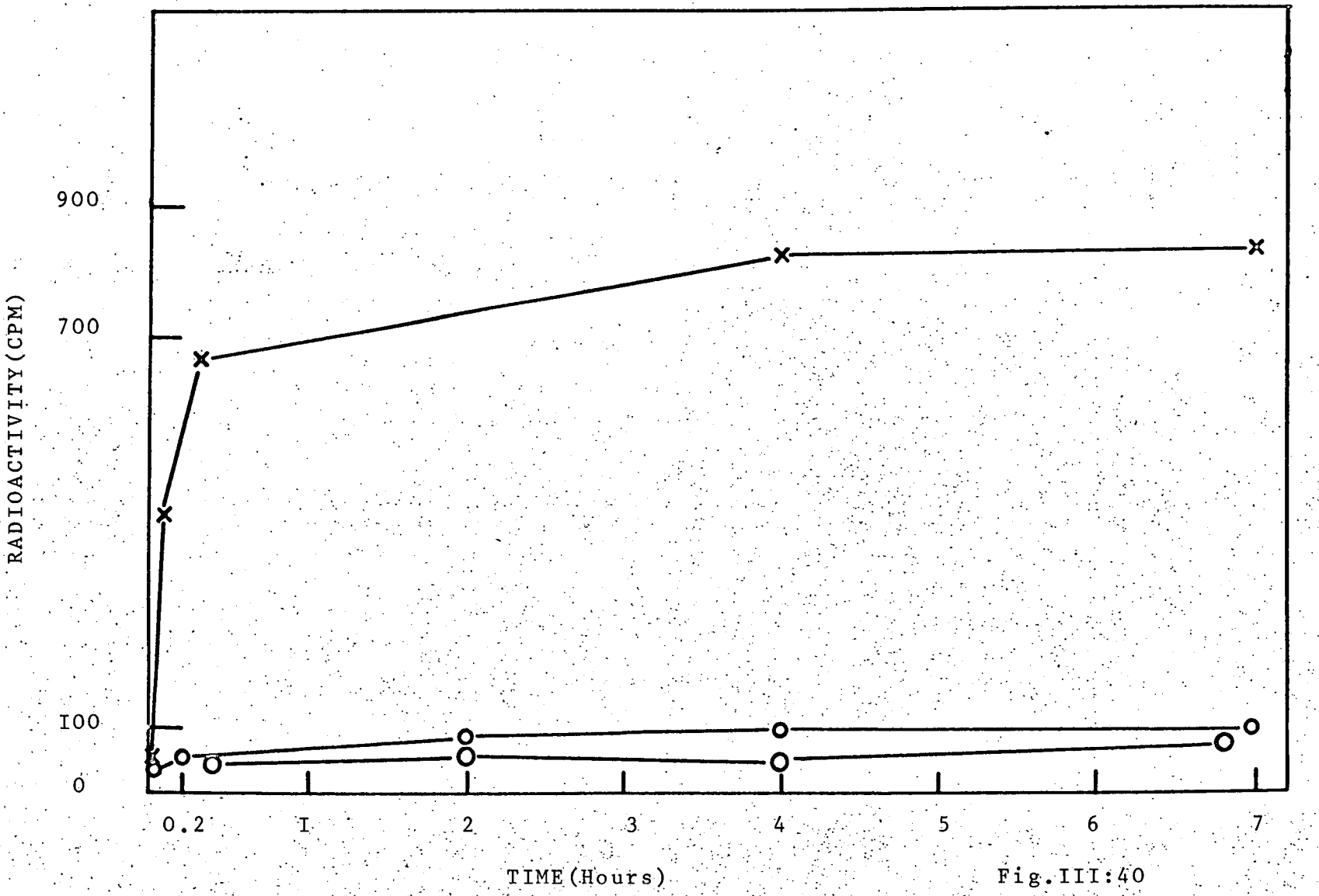


Fig. III:40

conditions (also Figure III:39) but there is the possibility that the Tm of 72°C may be a minimal value. An estimate of the efficiency of in situ hybridisation can now be obtained from the saturation value as shown in Figure III:40. This is calculated as follows. The number of cells fixed to the coverslip is 5×10^4 and since the amount of DNA per nucleus is in the range of 6×10^{-6} µg the amount of DNA per coverslip is therefore approximately 3×10^{-1} µg. The percentage of the mouse genome that comprises the light satellite DNA is 10% (Table III:13; also Waring and Britten, 1966) thus the amount of satellite DNA per coverslip is approximately 3×10^{-2} µg. The specific activity of the mouse satellite cRNA is 1.4×10^7 cpm/µg, and allowing for mainly single DNA strand hybridisation (Figure III:22) the amount of cRNA bound per 1.5×10^{-2} µg satellite would be 2.1×10^5 cpm. The efficiency of the scintillation counter is 10% in this case (as determined by counting specific volumes of samples of ^{3}H-ATP of known specific activity) giving a theoretical hybridisation value, at saturation, of 2.1×10^4 cpm/coverslip. The actual hybridisation value is around 9×10^2 cpm thus giving an efficiency of 4-5%.

DISCUSSION

The results of the experiments described above put the efficiency of in situ hybridisation at about 4-5%. Since this estimate is based on several assumptions it may be that this is a minimum value. For instance if the efficiency of the scintillation counting is actually lower than 10%, then the in situ hybridisation will be higher (actually the results of experiments with ^{3}H-uridine labelled cells do suggest it is about 10% (not shown)). Equally if the cRNA did not hybridise to

all the cells either because of insufficient excess or unavailability of some cells on the coverslip, the in situ estimate would be lower than it actually is. There have been other attempts to estimate the efficiency of in situ hybridisation. For instance, Jones (1970) hybridising mouse satellite DNA to the centromeric satellite on mouse chromosomes estimated about 10%; Steffensen and Wimber (1970) calculated the efficiency of hybridisation to the chromosomal 5S genes in Dipteran polytene chromosomes to be between 3-6%; and Jones, Bishop and Brito-da-Cunha (1973), analysing the complex formation between polyU and chromosomal bands of the polytene chromosomes of Rhynchosciara, suggested about 5-6% efficiency. 7% was calculated for the hybridisation of ribosomal RNA to the cap region of the Xenopus oocyte (Jones, 1973); and a lower value of 1-2% was calculated for the hybridisation of short-pulse-labelled RNA to Hela cell nuclei (John et al., 1969). Recently, Szabo et al. (1975) have suggested about 1% efficiency even under optimal conditions. All these estimates therefore fall into the range of 1-10% and are therefore in agreement with the 4-5% estimate derived here. The particular value of the method of estimation applied here is that at no stage is there an autoradiographic efficiency estimate which needs to be taken into account. In contrast, all other in situ hybridisation efficiency estimates have relied on assuming an autoradiographic efficiency estimate of 10% (e.g. Jones, 1970). This is an assumption, of course, since autoradiographic efficiency depends on a variety of factors including the thickness of the emulsion, the section or cell thickness, the choice of isotope, and various emulsion factors (Rogers, 1969). For tritium and a maximum section thickness of less than 5μ the auto-

radiographic efficiency is around 10-15%. The results presented here, however, suggest that the autoradiographic efficiency estimate is reasonable.

Another important feature of the method of estimation described here is the fact that the identity of the cRNA-DNA hybrids can be examined. Thus the mouse satellite cRNA-DNA hybrids formed at the cellular level possess similar thermal melting characteristics as those formed in RNA excess experiments (see Figure III:39; and Table III:13). The ability to do this may have important applications for other in situ hybridisation experiments.

However, it does remain that the efficiency of in situ hybridisation is relatively low. This may reflect the fact that in situ hybridisation reactions may not go to completion, the rate also appearing to be slower than conventional hybridisation reactions (see discussion on pg. 109). Consistent with this suggestion is the fact that the hybridisation of mouse satellite cRNA to cells fixed onto coverslips also seems rather slow (see Figure III:40). Recently, Szabo et al. (1975) also report that for some RNA species the rate of in situ hybridisation also appears to be slower than conventional hybridisation.

Clearly, the finding that the efficiency is low, together with the fact that in situ hybridisation can underestimate the amount of potential annealing of nucleic acid sequences if the results of conventional RNA excess hybridisation experiments are not considered (see Section B), indicates that for optimal resolving power the cytological hybridisation procedure should be carried out with RNA excess hybridisation data in mind.

The findings in this section are utilised in the designing of *in situ* hybridisation reactions involving Adenovirus cRNAs and are also considered in the light of interpretation of such experiments.

SECTION V

DETECTION OF VIRUS DNA SEQUENCES IN ADENOVIRUS-INFECTED, TRANSFORMED AND TUMOUR CELLS

RESULTS

1. cRNA excess hybridisation

cRNAs to Adenovirus 2, 7 and 12 DNAs were hybridised to Adenovirus transformed and tumour DNAs in conditions of cRNA excess, and under incubation conditions that were optimum for individual cRNA-homologous DNA reactions (see Section III, this Chapter). The reactions were terminated after several $t\frac{1}{2}$s for individual cRNAs, which is a reasonable measure of hybridisation time since the amount of complementary DNA on the membrane filters does not affect the rate of the base-pairing (Birnstiel et al., 1972). Saturation values were obtained for each hybridisation reaction together with control values obtained by hybridising Adenovirus cRNAs to rat DNA, mouse DNA, human DNA, and bacterial DNAs.

The results are shown in Table III:14. Except for two cases of Adenovirus 2 tumours, the saturation values for transformed DNAs and tumour DNAs are not consistently above the levels of heterologous controls. This suggests that in these cases Adenovirus DNA sequences are present only a few times per individual cell. Adenovirus DNA is nevertheless present in Adenovirus transformed cells and tumours since, for example, Adenovirus-specific antigens exist (Gallimore, 1972; Freeman et al, 1967; Green, 1970). The failure to detect them may therefore be due to limitations set by the RNA excess hybridisation technique (see Section III).

Although the amount of cRNA hybridising to a given amoung of complementary virus DNA can be calculated on the basis of saturation values (see Section III), a reconstruction experiment was performed. A known amount of virus DNA was loaded onto membrane filters together with heterologous carrier DNA (M. luteus) and the DNA challenged with the homologous virus cRNA. The result is shown in Figure III:41a.

The analytical complexity of Adenovirus DNA is around 25×10^6 daltons (Green, 1970) and the analytical complexity of the haploid genome of the rat is 1.8×10^{12} (Steele, 1968). From the specific activity of the virus cRNAs (1.7×10^7 cpm/μg) it can therefore be calculated that for 10^{-5} μg of virus DNA/1 μg transformed or tumour DNA, approximately 50-80 cpm hybridised would be expected. Figure III:41a shows, on extrapolation, that the actual hybridisation value is less than this. This low value can be increased by increasing the amount of DNA loaded onto individual membrane filters. Because of loading difficulties, however, 20μg of DNA represents an upper limit. For 20μg of transformed or tumour DNA there would theoretically be approximately 200-300 cpm/filter for 1 complete copy of Adenovirus DNA per diploid quantity of host DNA. The background levels of radioactivity are around this value (Figure III:41b; Table III:14). In fact, about two-three complete copies of the virus genome per diploid quantity of host DNA could actually be detected with reasonable confidence. If the transformed or tumour DNA possessed less than this amount of DNA sequences complementary to the cRNA then they would likely to undetected.

The situation is further complicated by the fact that only selective regions of the Adenovirus templates appear to be transcribed in vitro. Since RNA excess hybridisation is directly influenced by the

Table III:14

DNA(20μg)	cRNA	cpm/hybridised[a] (T.OPT. for each Reaction, 6xSSC 30% FA, several $t\frac{1}{2}$s)	Estimated virus DNA copies (calculated assuming 1.8×10^{12}d for haploid rat DNA and 25×10^{6}d for Ad DNA)
Ad2/REB/50p/B1	Ad2	292	< 2
Ad2/REB/10p/B1	Ad2	276	< 2
Ad2/T4	Ad2	300	< 2
Ad2/T5	Ad2	830	2-3
Ad2/T6	Ad2	900	3-4
Ad7/1	Ad7	295	< 2
Ad12/T1	Ad12	347	< 2
Rat	Ad2	280	
Rat	Ad7	380	
Rat	Ad12	351	
Mouse	Ad2	300	
Mouse	Ad12	256	
Human	Ad7	343	
Human	Ad2	370	
Human	Ad12	253	
E. coli	Ad2	219	
E. coli	Ad12	306	
E. coli	Ad7	256	
E. coli	Ad7	256	

[a] Average of four experiments

Figure III:4I. a) Hybridisation of Adenovirus I2 cRNA with increasing amounts of Adenovirus I2 DNA. Conditions of hybridisation:6xSSC 30% FA,50°C,several $t_{\frac{1}{2}}$s.IO :I cRNA:DNA.cRNA= I.7 xIO^{7}cpm/ug.b) Hybridisation of normal rat DNA with Adenovirus I2 cRNA.Conditions as for a).Background cpm. for M.Luteus DNA alone have been subtracted.

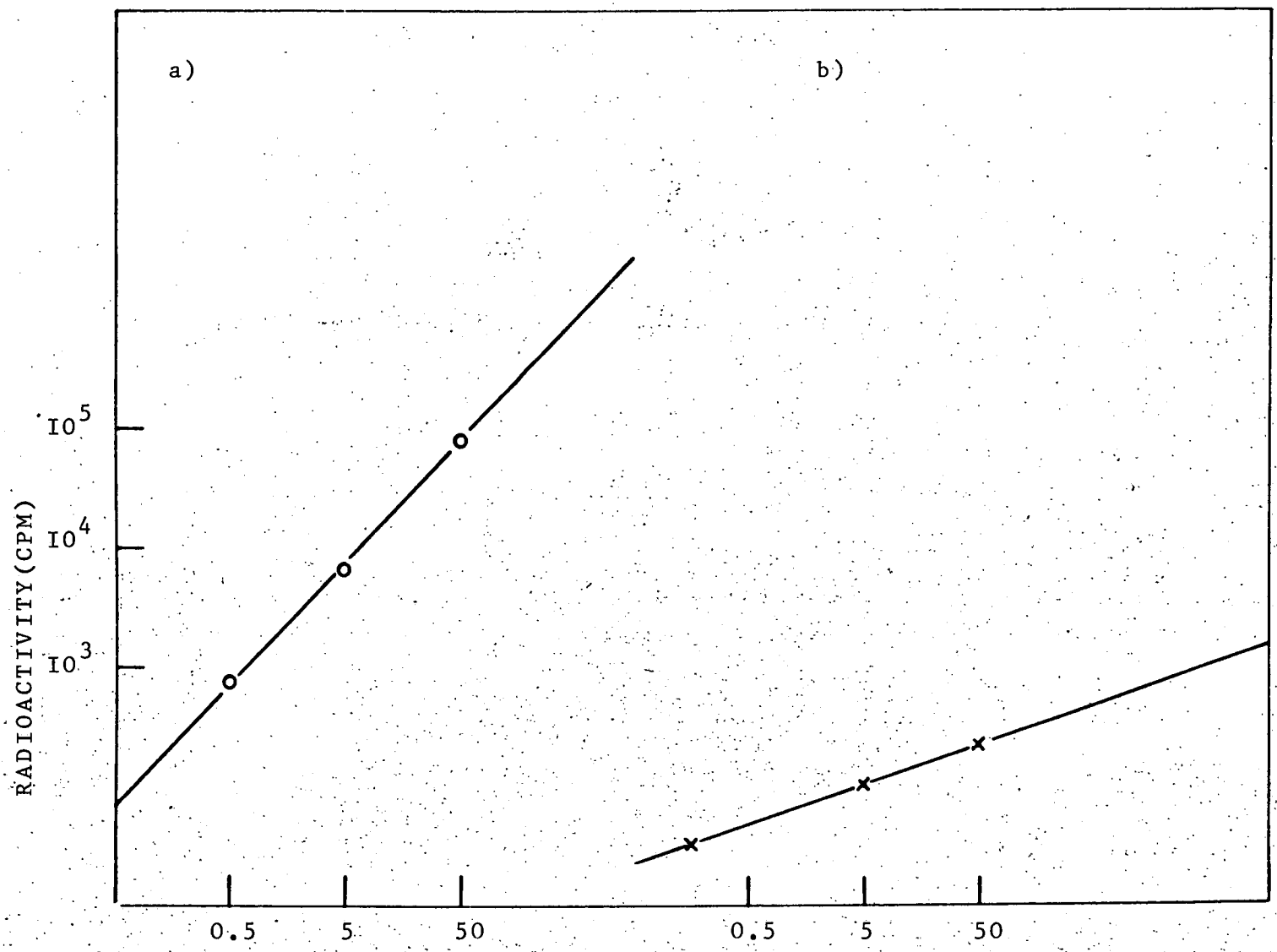

a)
b)
RADIOACTIVITY (CPM)
10^5
10^4
10^3
0.5
5
50
0.5
5
50
ng Adenovirus I2 DNA
ug rat DNA.

Fig.III:4I.

largest concentration of RNA sequences in the reaction (Bishop, 1972; Section III, this Chapter) it is clear that Adenovirus-specific DNA sequences would go undetected if they were not complementary to the greater proportion of RNA sequences in the cRNAs. For instance, cells could contain multiple copies of virus DNA sequences which are under-represented in the cRNAs and also contain less than three copies of sequences complementary to the greater proportion of the sequences in the cRNA. In this case, virus DNA would be present but would be undetectable.

Two Adenovirus 2 tumours, however, appear to react with Adenovirus 2 cRNA. One of them (Ad 2/T6) was analysed further. DNA was centrifuged in a neutral CsCl gradient and fractions hybridised with Adenovirus 2 or 12 cRNA. There was no hybridisation with 12 cRNA but 2 cRNA reacted (Figure III:42). A peak of radioactivity is observed at a buoyant density position of 1.706 gm/cm^{-3}, corresponding to a GC content of 43% (Sueoka et al., 1968). The buoyant density of Adenovirus 2 DNA is 1.716gm/cm^{-3} (Section I, this Chapter; also see Green, 1970) so that hybridisation cannot be to complete isolated virus DNA molecules. Adenovirus 2 cRNA was also hybridised across a CsCl gradient containing M. luteus DNA, rat DNA, and a trace amount of Adenovirus 2 DNA (Figure III:43). The cRNA hybridises to DNA sequences possessing a buoyant density of 1.715-1.716gm/cm^{-3} which is the buoyant density of Adenovirus 2 DNA.

Hybridisation in Figure III:42 therefore represents annealing of cRNA to either incomplete copies of Adenovirus genomes, or to regions of Adenovirus DNA which have become integrated into the rat DNA. These regions must at least correspond to those selectively transcribed in vitro by the E. coli RNA polymerase.

FigureIII:42. Hybridisation of Adenovirus 2 cRNA to Adenovirus 2 tumour DNA(Ad2/T6). cRNA(S.A. $I.7 \times IO^{7}$ cpm/ug) in IO:I excess over DNA sequences(theoretical);6xSSC 30%FA, several $t_{\frac{1}{2}}$s,50^{o}C.Peak of radioactivity corresponds to buoyant density of $I.705-I.706 gm/cm^{-3}$.

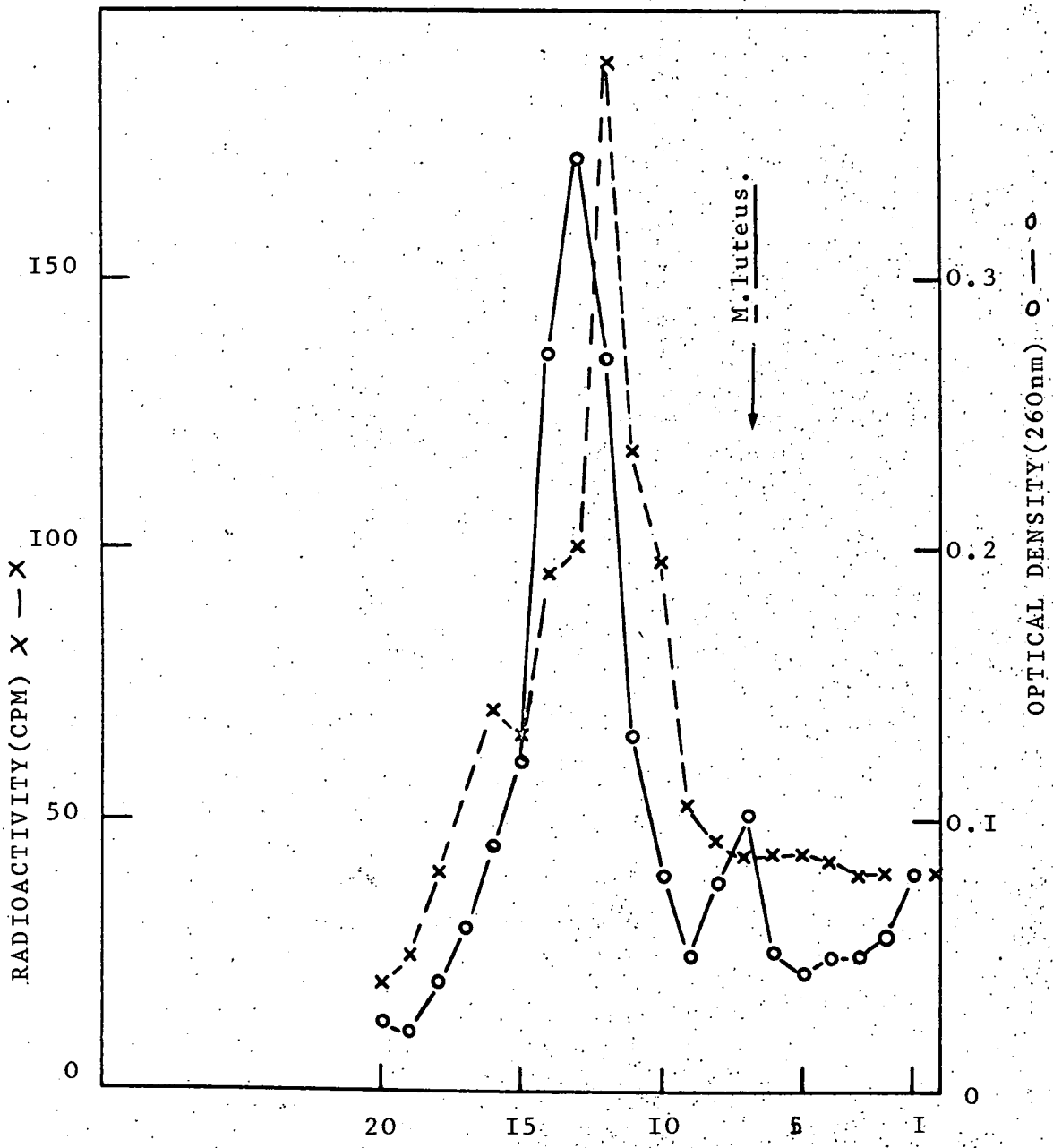

RADIOACTIVITY(CPM) X — X
150
100
50
0
OPTICAL DENSITY(260nm) o — o
0.3
0.2
0.1
0
M. luteus.
20
15
10
5
1
CsCL FRACTIONS.

Fig.III:42.

Figure III:43. Hybridisation of Adenovirus 2cRNA to CsCl gradient containing normal rat DNA and Adenovirus 2 DNA. M. luteus DNA included as a density marker (I.73 gm/cm^{-3}). Radioactivity is concentrated over fractions with buoyant density of I.7I5-I.7I6 gm/cm^{-3} which is the buoyant density of Adenovirus 2 DNA in neutral CsCl (see Table III:I). Conditions of hybridisation as for Figure III:42.

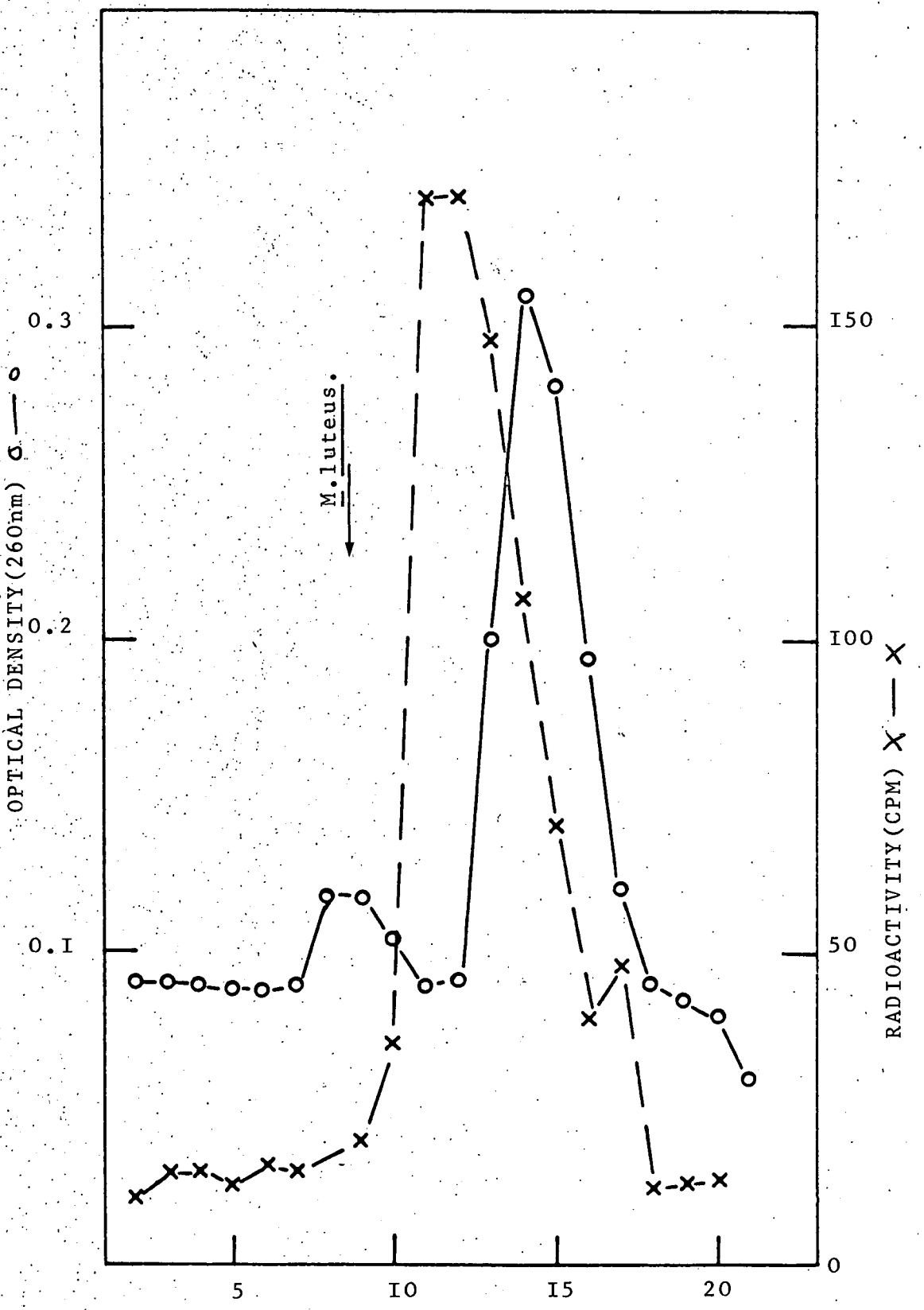

0.3
0.2
0.I
OPTICAL DENSITY(260nm) o — o
M.luteus.
I50
I00
50
0
RADIOACTIVITY(CPM) X — X
5
I0
I5
20
CsCl FRACTIONS

Fig.III:43.

The amount of virus DNA in this particular tumour DNA can be roughly assessed from the saturation value (Table III:14). About 3-4 copies of virus DNA sequences which are complementary to the cRNA are present. This may be a minimum estimate of total virus DNA sequences, however, since there may be sequences present which are not complementary to the greater proportion of the RNA sequences in the cRNA.

In the case of the Adenovirus 2 tumour (Ad2/T5) there are approximately 2-3 copies of virus DNA sequences complementary to the virus cRNA (also Table III:14).

2. DNA excess hybridisation

A DNA excess ratio of approximately 100:1 was calculated. Hybridisation was carried out at 65°C in 2 x SSC over a wide range of Cot values up to 10^4. DNA was at a concentration of 10mg/ml and cRNA at 10^{-3} µg/ml (1.7×10^7 cpm/µg). The results are shown in Table III:15. The transformed DNAs are negative. One Adenovirus 2 tumour DNA (Ad2/T5) is positive. (The Ad2/T6 tumour was not analysed by the DNA excess method because there was not sufficient DNA.)

cRNA transcribed from a total DNA template (rat) hybridises to its template DNA to about 50% while the Ad2/T5 tumour DNA hybridises to Adenovirus 2 cRNA to about 40% (Figure III:44;Table III:15). Failure to achieve full hybridisation, even when expected (Figure III: 44) may be due to not sufficient DNA excess, breakdown of the cRNA over prolonged incubation times (Bishop, 1972a; Campo, 1973) or RNase sensitivity of the hybrids (Bishop, 1972b). For this reason it cannot be accurately judged whether incomplete hybridisation to the tumour DNA represents inherent limitations on the technique, or the fact that some cRNA sequences are not represented in the virus DNA

in the tumour. If a small percentage of the cRNA sequences do represent all the Adenovirus 2 genome as suggested by Pettersson et al. (1974), failure to achieve full 100% hybridisation could be due to the fact that some virus DNA sequences are missing in the tumour DNA. The actual amount of the virus DNA sequences complementary to the cRNA can be roughly assessed from the $Cot\frac{1}{2}$ for the hybridisation reaction. The frequency(F) of these DNA sequences is calculated from the formula

$$F = \frac{Cot\frac{1}{2}\text{hybridisation}_{\text{E. coli cRNA (standard)}}}{Cot\frac{1}{2}\text{hybridisation}_{\text{Tumour (Ad2/T5)}}} \times \frac{C(\text{Ad2/T5 tumour})}{C(\text{E. coli})}$$

where C = analytical complexity of haploid DNA (Bishop, 1972a; Melli et al., 1971).

Thus

$$F = \frac{15.9(\text{Melli et al., 1971})}{Cot\frac{1}{2}\text{ hybridisation Tumour (Ad2/T5)}} \times \frac{1.8 \times 10^{12}}{2.7 \times 10^{9}}.$$

The $Cot\frac{1}{2}$ hybridisation for Ad2/T5 DNA-Adenovirus 2 cRNA is 6.6×10^{3} (Figure III:44). Therefore F = 2-3 for Adenovirus 2 DNA in the Ad2/T5 tumour. Thus DNA excess hybridisation gives 2-3 copies of virus DNA complementary to the Adenovirus 2 cRNA.

3. In situ hybridisation

Using cRNAs to Adenovirus DNAs, in situ hybridisation experiments were performed with Adenovirus transformed cells, Adenovirus and Adenovirus transformed cell-induced tumours, Adenovirus infected cells and cells devoid of Adenovirus DNA.

The results of Section IV demonstrated that it is best to perform an in situ localisation experiment at the optimal conditions of hybridisation for the particular RNA. These optimal hybridisation

Figure III:44. Hybridisation of cRNA to total DNA in DNA excess. 65°C in 2xSSC. DNA: IOmg/ml. cRNA at IO^{-3} ug/ml. (S.A. $I.7xIO^{7}$ cpm/ug).
o-o rat cRNA transcribed from a total rat DNA template (E.coli RNA polymerase) hybridised to total normal rat DNA (liver).
x--x Adenovirus 2 cRNA hybridised to Adenovitus 2 tumour DNA (Ad2/T5).

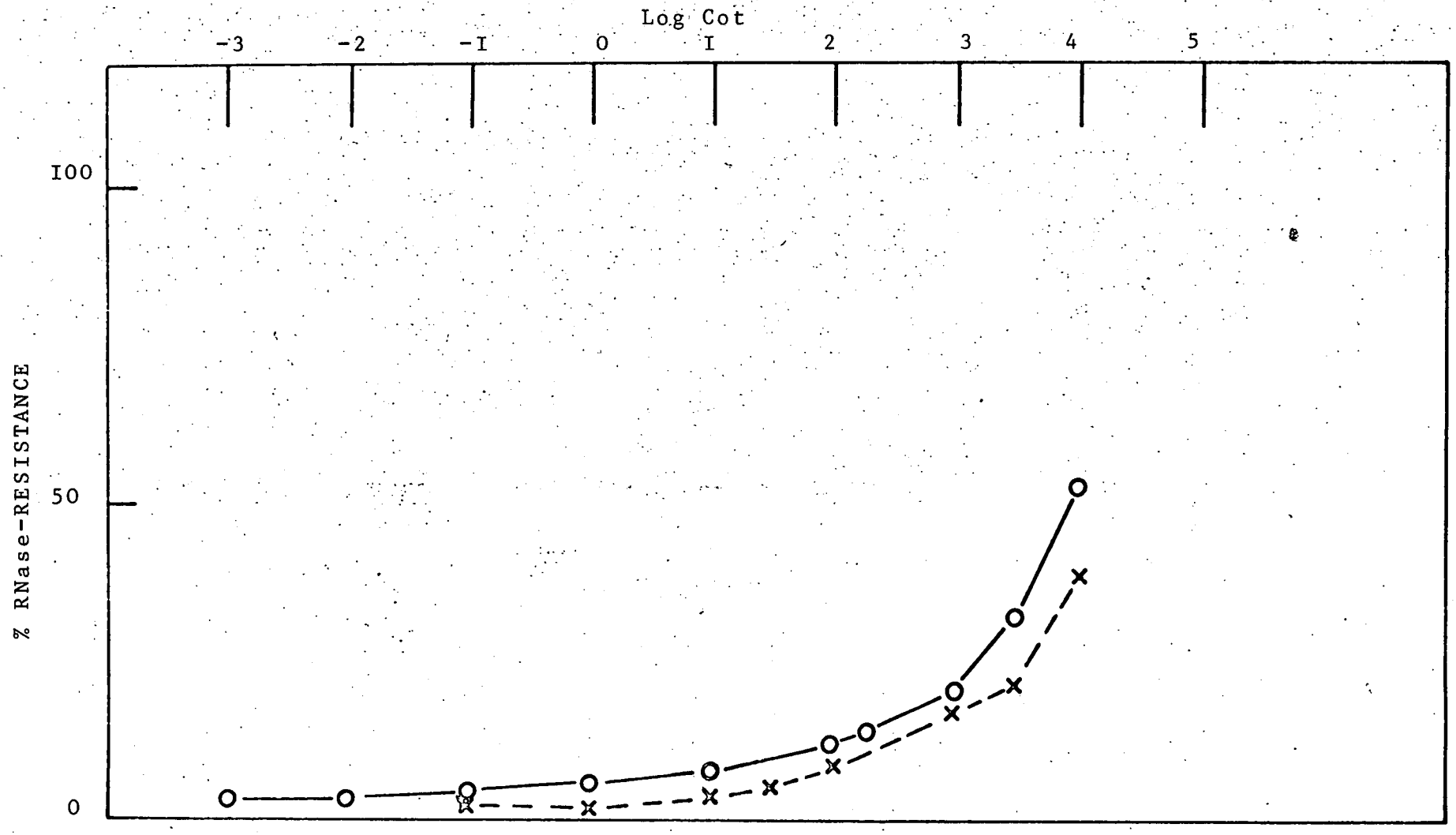


Fig.III:44.

Table III:15

DNA*	cRNA	**%RNase-resistance at Cot 10^4
Ad2/REB/50p/B1	Ad2	< 5
Ad2/REB/10p/B1	Ad2	< 5
Ad2/T4	Ad2	< 5
Ad2/T5	Ad2	39
Ad2/T6	Ad2	nd
Ad7/1	Ad7	< 5
Ad12/T1	Ad12	< 5
Rat	Ad2	< 5
Rat	Ad12	< 5
Rat	Ad7	< 5
Rat	Total Rat	50

* DNA excess of approximately 100:1 (calculated virus DNA sequences)

** %RNase-resistance for cRNA-cRNA annealing subtracted [see Table III:6]

conditions for Adenovirus cRNAs have been studied in Section III. The *in situ* hybridisation experiments described below were therefore conducted with these considerations in mind.

a) Permissive cells.

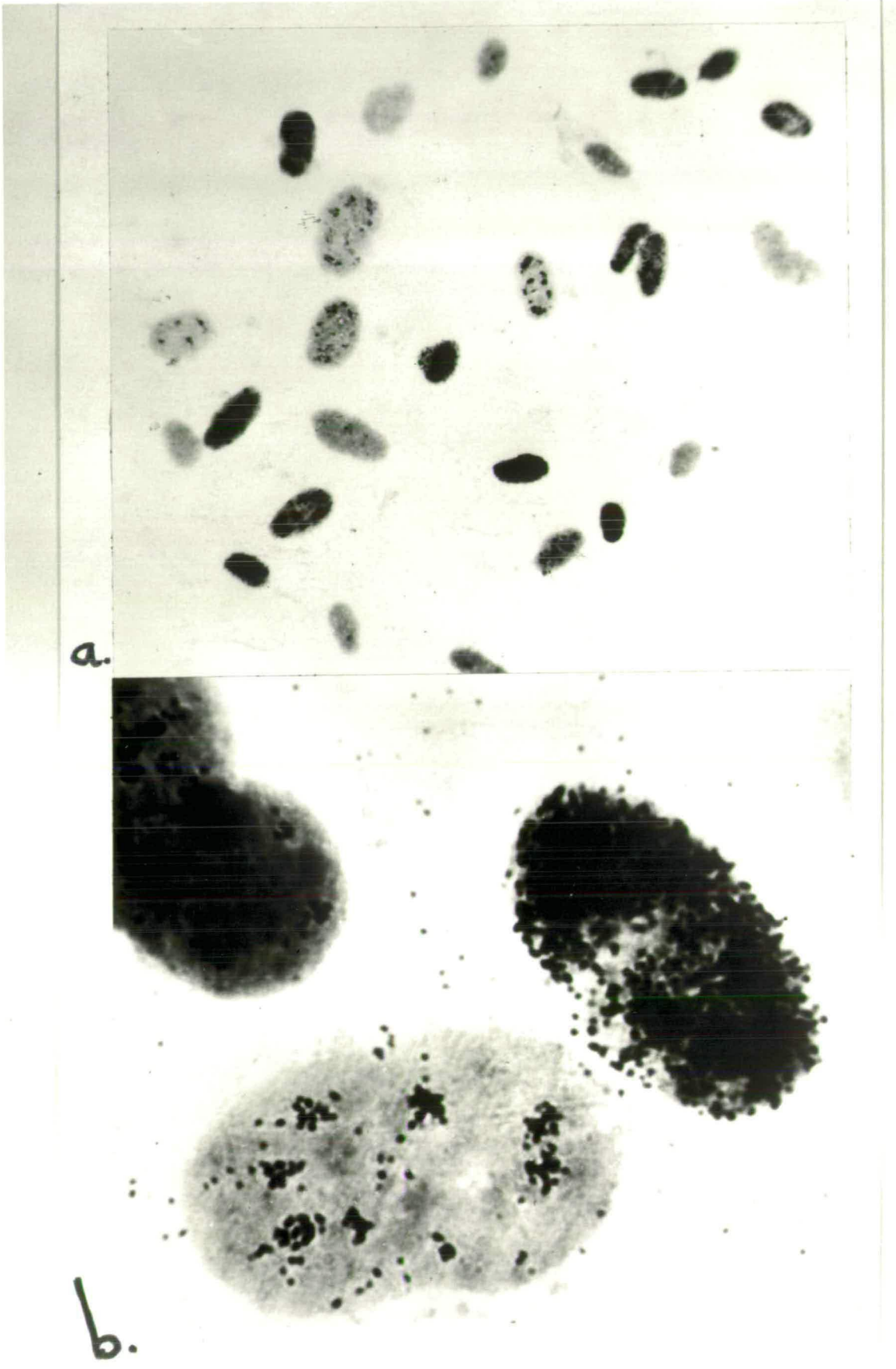
HEK cells are permissive for the replication of Adenovirus DNA and the subsequent production of virus particles (e.g. Ledinko and Fong, 1969; Zur Hausen, 1967; McDougall, 1971). Hela cells are also permissive for the replication of Adenovirus. Adenovirus 12 replication was studied in HEK cells; Adenovirus 2 replication was studied in HEK cells and Hela cells.

1. HEK cells were infected at multiplicities of 100pfu/cell with Adenovirus 12. At 48 hours post-infection the cells were hybridised with Adenovirus 12 cRNA, Adenovirus 2 cRNA and Adenovirus 7 cRNA. In the case of the homologous reaction there are pools of grains which are restricted to the nuclei of the cells (Figure III:45). The stage of replication of virus DNA in different nuclei is variable, suggesting asynchrony in initiation of replication or rate of replication. This may be due to asynchrony in virus penetration, or asynchrony in some cell-mediated function.

Grain pools in Adenovirus 12 infected HEK cells have also been detected by McDougall *et al* (1972) and Dunn *et al*., (1973). Similar pools have also been observed by Watkins (1973) who, using *in situ* hybridisation with SV40 cRNA, showed there was a focal distribution of SV40 replicating DNA within the nuclei of a small percentage of baby rabbit kidney cells transformed with SV40 virus.

Hybridisation of Adenovirus 2 or 7 cRNA to Adenovirus 12 infected cells was negative. In view of the fact that infected cells possess

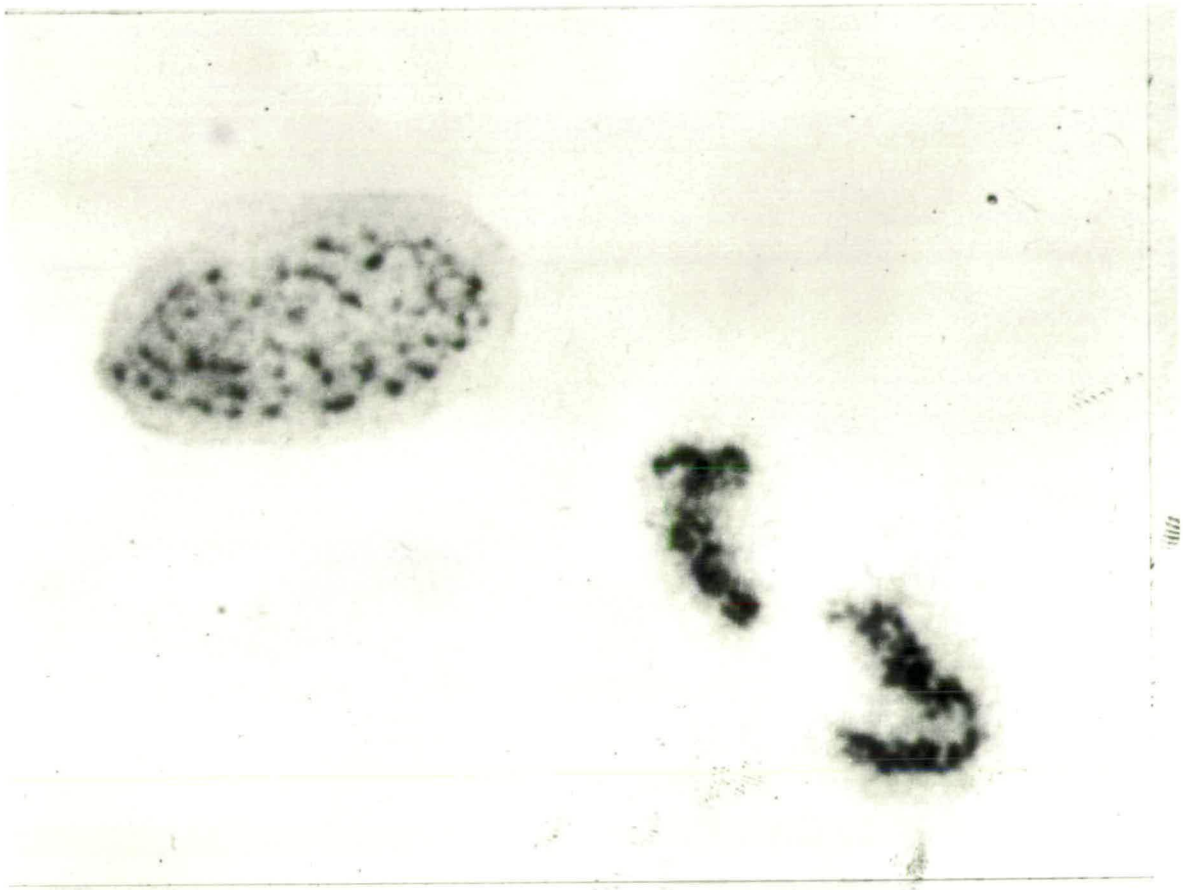
Figure III:45. *In situ* hybridisation of Adenovirus I2 cRNA and Adenovirus I2-infected HEK nuclei. Cells were originally infected at IOOpfu/cell and harvested at 48 hours post-infection when nuclear preparations were made. Conditions of hybridisation :65°C in 2xSSC;0.Iug cRNA/4ul;I6 hours. S.A. of cRNA=I.7 xIO^{7} cpm/ug. Exposure time:4 weeks (Ilford K2 emulsion). Pools of grains represent pools of replicating virus DNA.

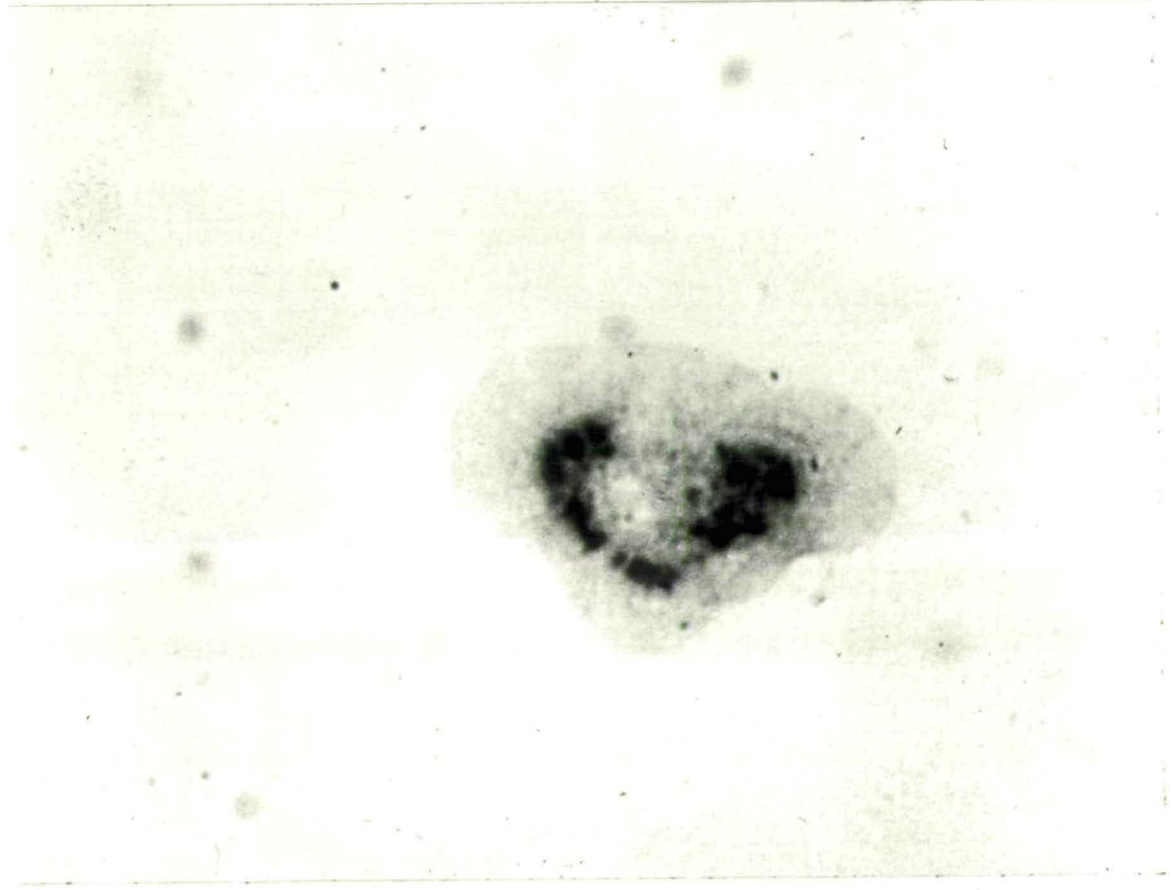

a.
b.

a great number of virus particles at 48 hours post-infection and a large amount of excess virus DNA which appears to remain unassembled into virus particles (Green et al., 1970), together with the finding that Adenovirus 12 DNA shares 20-24% of its base-sequences with Adenovirus 2 DNA and 1o-25% with Adenovirus 7 DNA (Green, 1970), the lack of cross-hybridisation seems unexpected. However, Adenovirus cRNAs are selectively transcribed from their template DNAs (Section III, Chapter III, this Thesis; Green and Hodap, 1972; Dunn et al., 1973; Pettersson et al., 1974) in such a manner that the greater proportion of the cRNA sequences are not common to different serotypes. Thus the lack of cross-hybridisation at the cytological level is explicable.

Intranuclear inclusions are a general feature of Adenovirus infected cells (Ginsberg and Dingle, 1965). For example they appear regularly in Adenovirus-infected human cells (Boyer et al., 1959; Morgan et al., 1957; Phillips and Raskas, 1972), and in canine cells infected with Canine Adenovirus (Yamamoto and Shahrabadi, 1971; Shahrabadi et al., 1972). Their significance is not totally understood although histochemical and autoradiographical studies have shown that they are composed of DNA, protein and RNA (Yamamoto and Shahrabadi, 1971). Subsequent studies on the nature of the inclusion-associated DNA has demonstrated it to be virus-specific (Shahrabadi et al., 1972). Similar nuclear inclusions appear to be the cytological site of virus DNA replication in SV40 transformed cells which are semi-permissive for the virus (Watkins, 1973). For Adenovirus-infected cells this also may be the case since previous suggestions that the viral DNA is initially synthesised near the cell membrane appear to have been refuted (Simmons et al., 1974). An example of the intra-nuclear inclusions induced by

Figure III:46. Intranuclear inclusions in Adenovirus I2 -infected HEK cells. HEK cells have been infected at 100 pfu/cell with purified Adenovirus I2 and harvested 48 hours post-infection. Stained with Giemsa(pH 6.8) for 5mins. at room temperature(24^{o}C).



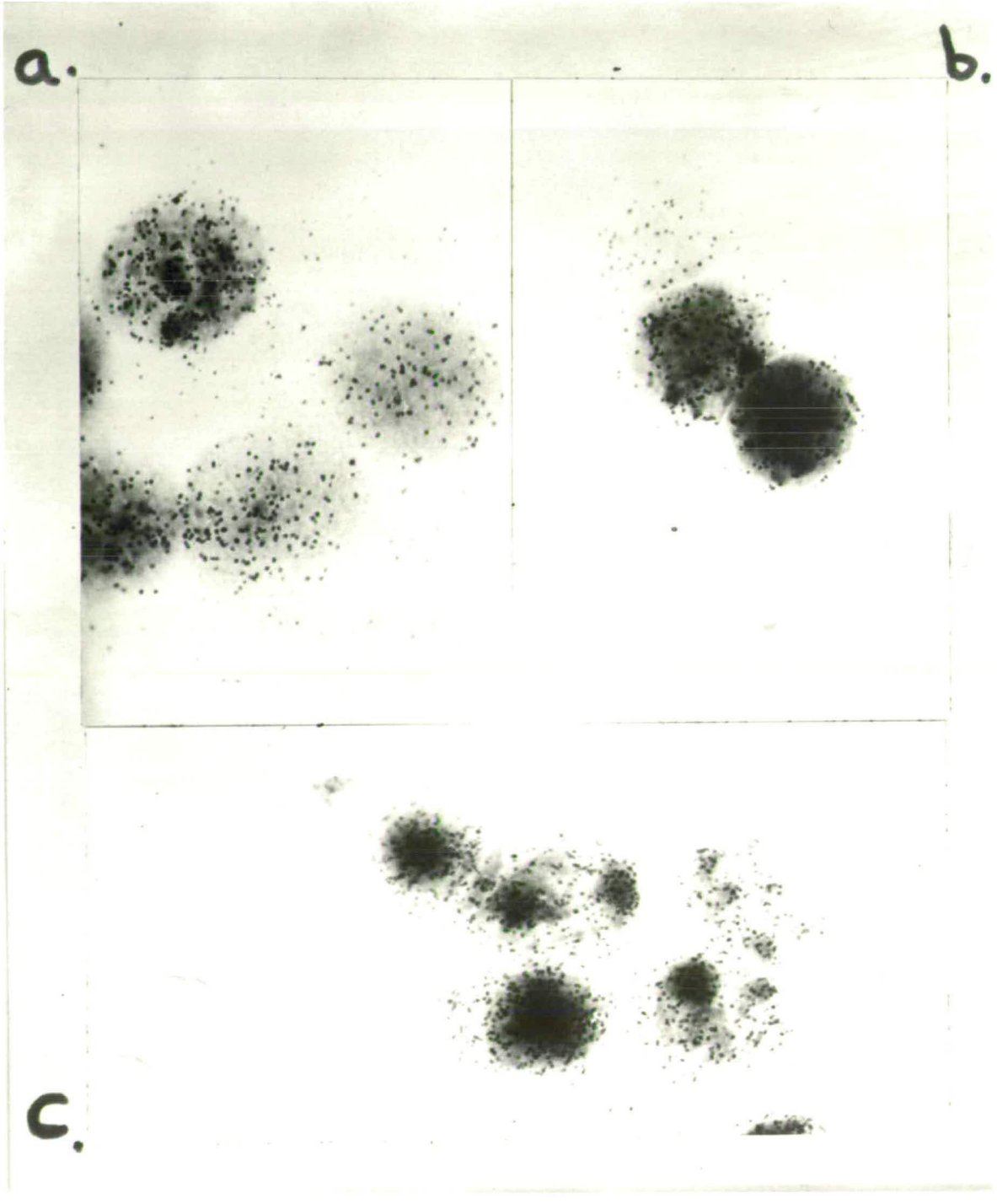


Adenovirus 12 in human embryonic kidney cells is shown in Figure III: 46. As virus replication proceeds in these cells the inclusions become characteristically shaped within the nucleus. The varying degree of morphology of these inclusions clearly follows the pattern of grain pools seen in the infected cells hybridised with Adenovirus 12 cRNA. The pattern of viral DNA synthesis and the formation of the intranuclear inclusions are therefore similar in their nuclear distributions. This suggests that the giemsa-staining inclusions are directly involved with the replication of the viral DNA. Whether viral DNA synthesis is initiated within these regions is not known.

2. HEK cells were infected with Adenovirus 2 at a m.o.i. of 100pfu/cell and the cells harvested at 50 hours post-infection. Hela cells were infected with Adenovirus 2 at a m.o.i. of 5pfu/cell and the cells harvested 50 hours post-infection. In addition, Hela cells were also infected with Adenovirus 5 at a m.o.i. of 50pfu/cell and the cells harvested 50 hours post-infection. (Adenovirus 5 was a gift from Dr. J. Williams, Virology, Glasgow.) Hybridisation with Adenovirus 2 cRNA is positive (Figure III:47), with Adenovirus 12 or 7 cRNAs it was negative (not shown). Grains are present throughout individual nuclei of either Adenovirus 2 or 5-infected cells. Some cells were devoid of grains which suggests that virus DNA is either not replicating or is not present in these cells. These cells may not have been originally infected.

What is particularly interesting, however, is that the pattern of virus DNA replication, both in the Adenovirus 2-infected HEK and Hela cells, appears to be different from the pattern found for Adenovirus 12-infected cells (c.f. Figure III:45). Although there are grains distributed over the entire nucleus, frequently they are localised to nuclear areas (Figure III:48) which are clearly discrete

Figure III:47. *In situ* hybridisation of Adenovirus 2 cRNA to Adenovirus 2 and 5-infected Hela cells. Infected cells were harvested at 50hours post-infection(see text for details)and challenged with Adenovirus 2cRNA(S.A. I.7 xIO^{7}cpm/ug) at $65^{o}C$ for I6hours in 2xSSC.cRNA=0.0Iug/4ul.Exposure time 8weeks.(K2 emulsion).
a) and b) Adenovirus 2-infected Hela cells c)Adenovirus 5-infected Hela cells. Note dispersion of grains and frequent grain localisation to regions within individual nuclei.

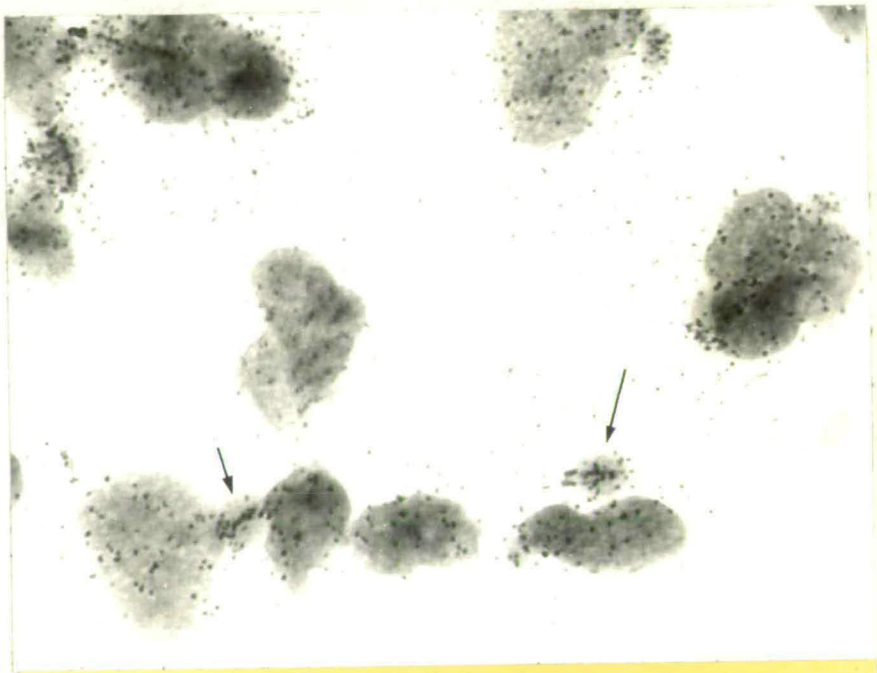

a.
b.
c.

and distinct since they stain differently to the rest of the nucleus with either Giemsa (Figure III:48 also) or with Methyl Green Pyronin (not shown). In some cells there are several areas or "bodies" which either surround or are contained within the nucleus. Furthermore, these differently-staining "bodies" appear to disperse around the periphery of certain nuclei and grains are then associated with the nuclear membrane (see Figure III:48d for example). The pattern of replication is clearly different from Adenovirus 12-infected cells.

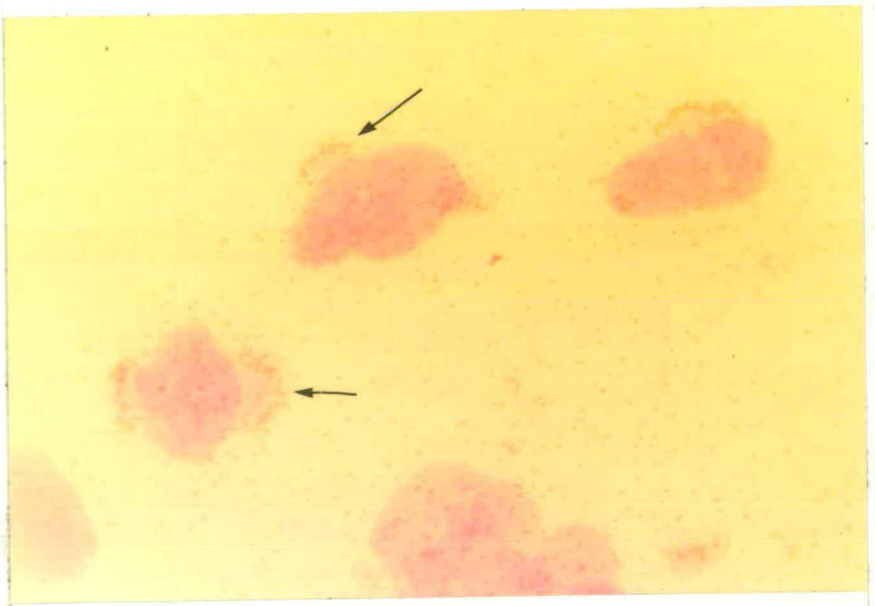
Adenovirus 5-infected Hela cells possess grains when hybridised with Adenovirus 2 cRNA (Figure III:47). The presence of Adenovirus 5 particles was determined by Electron Microscopy (Figure III:49): they clearly exist in the infected cells. Adenovirus 2 cRNA is therefore capable of hybridising with Adenovirus 5 DNA at the cytological level. Both these DNAs share considerable DNA-DNA homology, extending to 95% (Green, 1970). Moreover Adenovirus 2 cRNA hybridises with Adenovirus 5 DNA immobilised on membrane filters (Dunn, personal communication).

Again, however, what is particularly interesting is the finding that the pattern of Adenovirus 5 DNA replication, as monitored by Adenovirus 2 cRNA hybridisation, is unlike Adenovirus 12 replication but like Adenovirus 2 replication (c.f. Figures III:45; and III:47). Since Adenovirus 2 and 5 are more closely related to each other (they share the same subgroup) than to Adenovirus 12, and since the pattern of replication does not appear to be attributable to different cellular affects (Hela and HEK are similar), there is a strong possibility that the difference in virus DNA replication reflects a difference between Adenovirus 12 and Adenovirus 2 or 5.

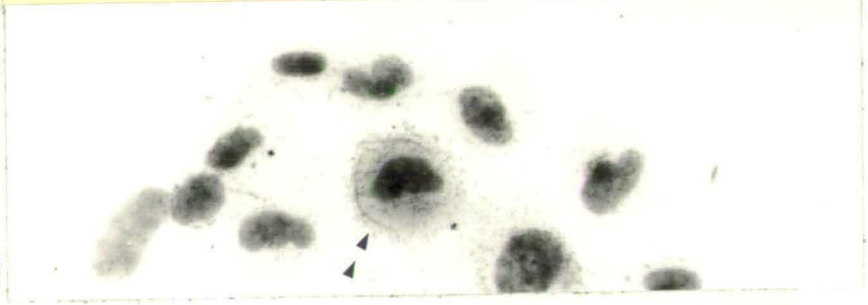
Figure III:48. *In situ* hybridisation of Adenovirus 2 cRNA to Adenovirus 2-infected HEK cells. Conditions of hybridisation same as for Figure III:47. In a considerable number of cases hybridisation is predominantly to "bodies" outside or within individual nuclei. (see a) and b)). Moreover in some cells hybridisation occurs mainly around the periphery of the nucleus (see arrow in d)), most likely reflecting the break up of the "bodies" and release of virus. In the colour photograph grains appear brown as a result of both overdevelopment and not enough post-fixation washing of the autoradiograph and the sensitivity of the colour film emulsion to red light.



a)



b)



d)

Figure III:49 also shows that at the E.M. level, virus is frequently concentrated in regions of the nucleus which appear to be "budding" off. These regions most probably correspond to the "bodies" detected at the L.M. level.

Whatever the significance of the above results are, they clearly show that virus-specific DNA sequences can be detected at the single cell level. The same approach was therefore adopted for Adenovirus transformed or tumour cells.

b) Transformed cells.

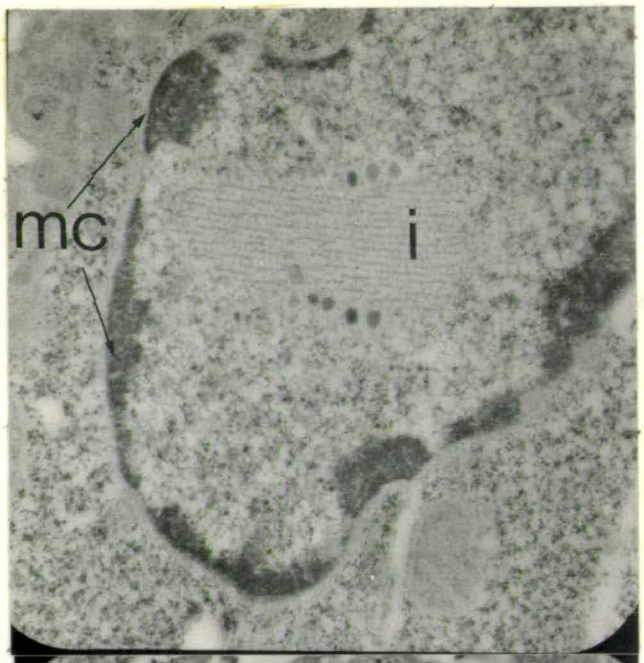
Adenovirus 2 and 7 transformed cells were examined for the presence of virus DNA. Table III:16 and Figure III:50 demonstrate that the numbers of grains present over these cells are low. Control cells derived from mouse, normal rat, human and toad (Xenopus laevis) tissue all possess similar amounts of grains. In other experiments no grains were observed for either the transformed cells or the control cells. Additionally, autoradiographs exposed for periods of six months to a year still did not show an increased number of grains in the transformed cells compared to the control cells (not shown).

c) Tumours.

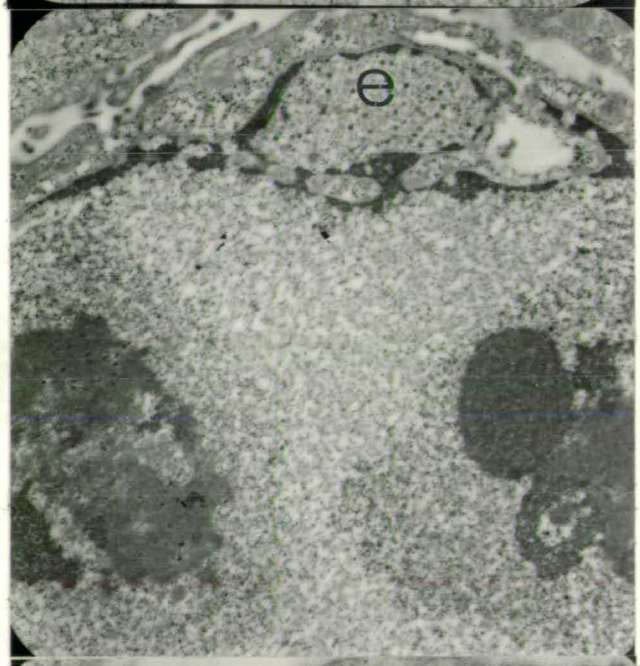
Newborn Hooded Lister rats were inoculated either with purified Adenovirus or with Adenovirus transformed cells. In most cases, the rats were previously immunosuppressed (Gallimore, 1972). Frozen sections and in vitro established cell lines were prepared as described in Materials and Methods.

Table III:16 summarises the results of in situ hybridisation with tumour cell lines: and Figure III:51 shows a typical in situ hybridisation to a frozen section. This frozen section was derived from

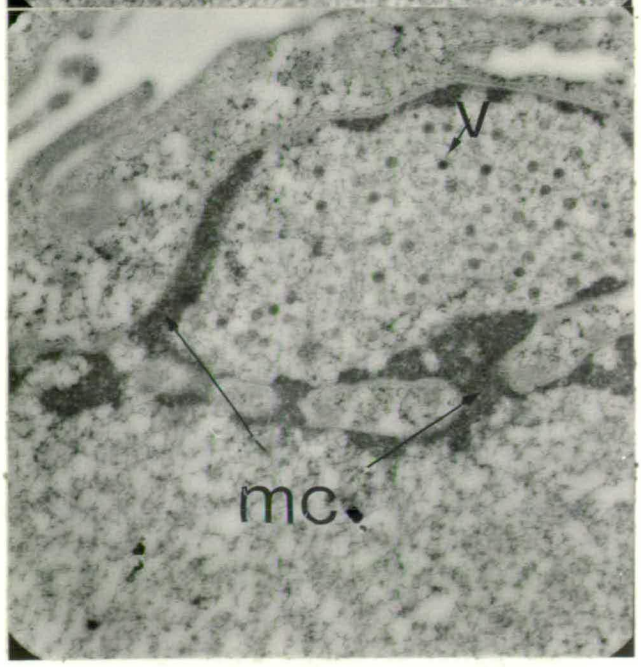
Figure III:49. E.M. sections of Adenovirus 5-infected Hela cells. Infected cells were prepared for E.M.sectioning as described routinely(GMA embedding).Thin sections (silver interference colour:800-1000 Å thick) were cut on a Poster Blum Ultra-microtome MK.2,stained doubly with Uranyl acetate and Lead Citrate,and examined in an AEI EM6 electron microscope with a double condenser.
a) E.M. section of a cell which contains a peculiar inclusion(i) common only to virus-infected cells. Virus-like particles are evident in an area of the nucleus which is delineated by the marginated chromatin (m.c.) which is also a feature of virus-infected cells.
b) Electrom micrograph showing newly-formed viruses in what appears to be a fold of the nuclear envelope(e).These constrictions are common to Adenovirus 5-infected cells and may correspond to the "bodies" seen at the L.M.level (see Figure III:48 and 46).c) Also note the marginated chromatin (m.c.) and one particular virus particle which possesses the characteristic nucleoid and capsid(v).



a) X 15,000

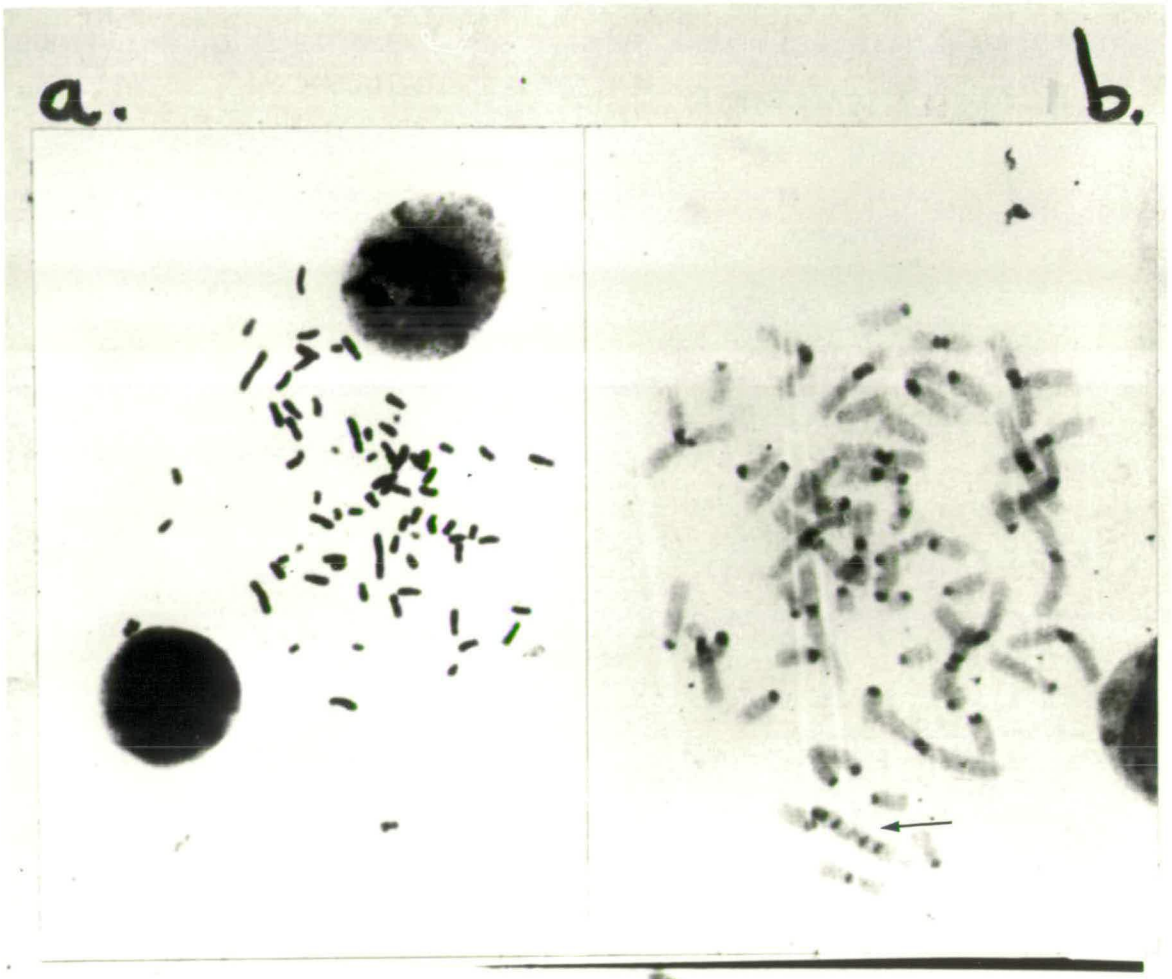


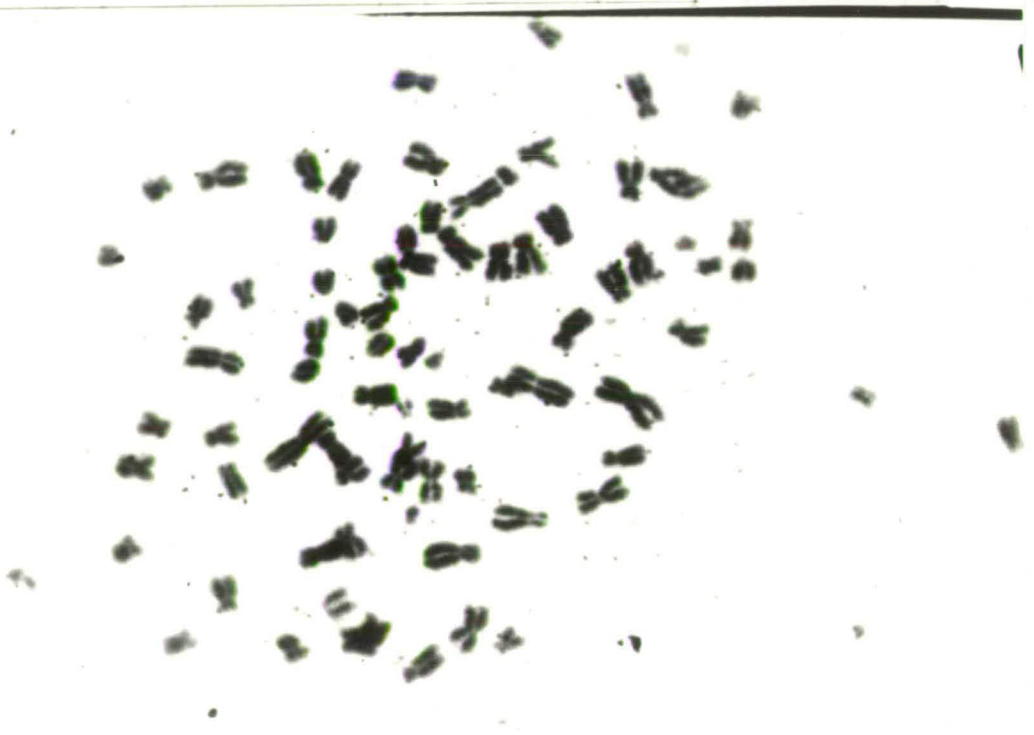
b) X 3,700



c) X 12,000

Figure III:5_0. In situ hybridisation of Adenovirus cRNAs to Adenovirus transformed cell chromosomes.Conditions of hybridisation: cRNA at 0.0Iug/4ul,2xSSC;I6hours at $65^{o}C$. S.A. of cRNA= I.7 xIO^{7}cpm/ug. a) Adenovirus 7 transformed hamster chromosomes(Ad7/I) challenged with Adenovirus 7 cRNA;b)Adenovirus 2 transformed rat cell chromosomes(Ad2/REB/IOp/BI) challenged with Adenovirus 2 cRNA;c) Adenovirus 7 transformed hamster cell chromosomes(Ad7/I) challenged with Adenovitus 7 cRNA. Exposure times:a) and b) 2 months.c)6 months. There are few grains.Even after 6 months exposure,grains are not associated in any specific way.Also note marker chromosome in Ad2/REB/IOp/BI line(arrow).





c.

the Adenovirus 2 tumour-Ad2/T5-which was induced by inoculating Adenovirus 2 transformed cells, originally infected at 50pfu/cell, into newborn rats (see Materials pg.23). It did not show any autoradiographic grains after *in situ* hybridisation with either Adenovirus 12 or 7 cRNAs (not shown). These results therefore suggest that hybridisation with Adenovirus 2 cRNA to this tumour section results in specific detection of Adenovirus 2 DNA at the cytological level. Another Adenovirus 2 tumour (Ad2/T4) did not possess any grains on *in situ* hybridisation with Adenovirus 2, 12 or 7 cRNAs (not shown). It therefore resembles the transformed cell line (Ad2/REB/10p/B1) used to produce it *in vivo* in that there does not appear to be any hybridisation of virus DNA and cRNA at the cytological level. The Adenovirus 12 induced tumour does not possess a higher number of grains than control cells (Table III:16).

These findings suggest that different tumours possess different amounts of Adenovirus DNA. There may be an alternative explanation however. RNA can anneal to complementary single-strand RNA molecules. For example, the denatured strands of phage MS2 or QB can reanneal and form well-matched double stranded RNA (Friedrich and Feix, 1972). RNA-RNA annealing also forms the basis of the measure of self-complementarity of cRNA preparations (Bishop, 1972a; Section III, this Chapter of this Thesis).

Adenovirus cRNAs would be capable of self-annealing to *in vivo* Adenovirus-specific RNA if, either the cRNAs were largely symmetrically transcribed *in vitro* or if the *in vivo* virus RNA was largely symmetrically transcribed. The cRNA is mainly asymmetrically transcribed (Section III, this Chapter; also Pettersson *et al.*, 1974). There is evidence, however, which suggests that a large percentage of virus-specific RNA

Table III:16

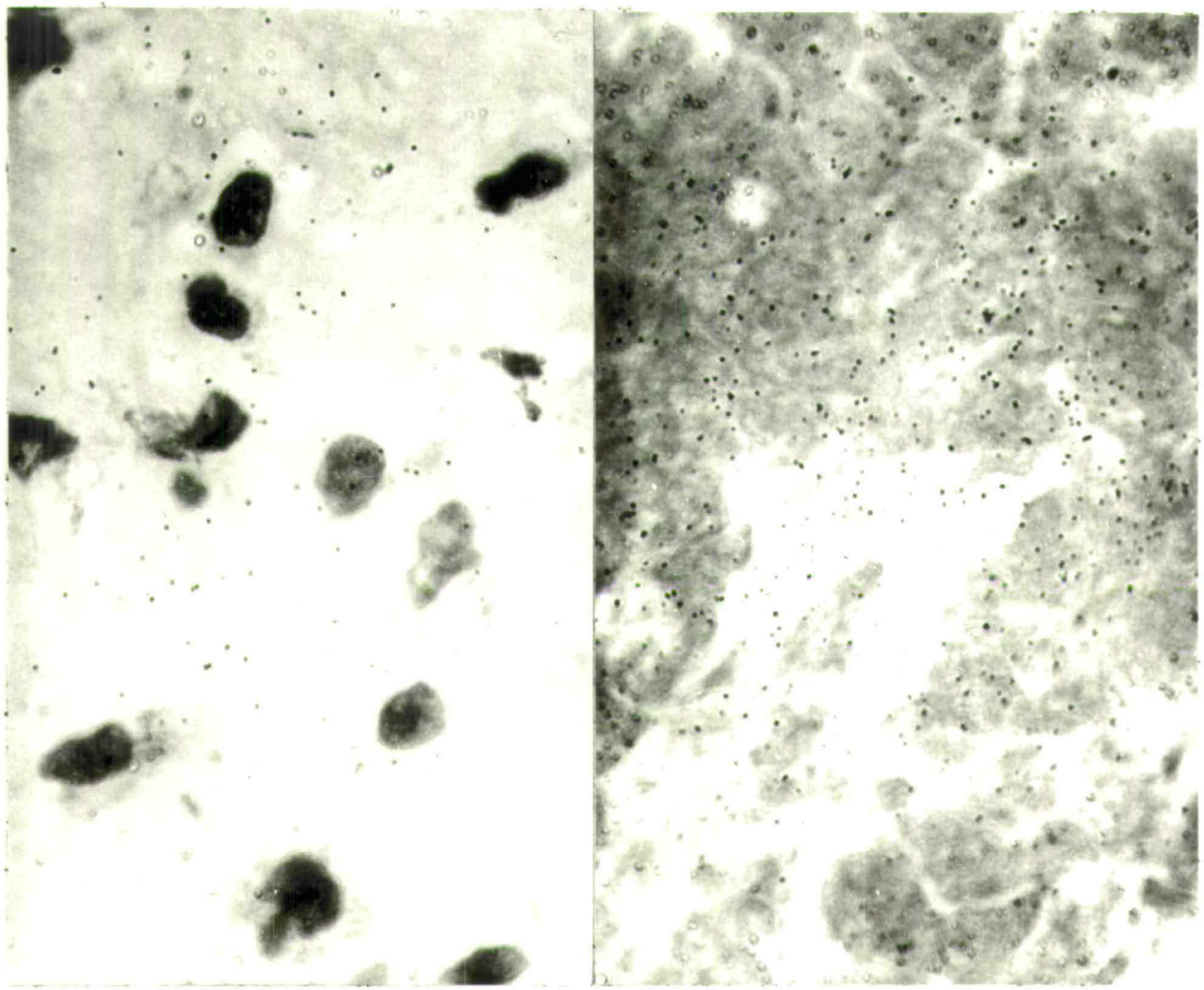
Cell	[b] cRNA	[c] average grain counts/nucleus or metaphase spread
Ad2/REB/10p/B1	Ad2	2
Ad2/REB/50p/B1	Ad2	4
Ad7/T1	Ad7	3
Ad2/T4	Ad2	3
Ad2/T5	Ad2	15
Ad2/T6	Ad2	nd
Ad12/T1	Ad12	4
[a] Ad2/T5	Ad2	3
rat	Ad2,7,12	2
mouse	Ad2,7,12	2
human	Ad2,7,12	3
toad	Ad2,7,12	2

[a] Denatured with 0.2N HCl and RNased (20µg/ml for 60 mins. at room T°C) prior to in situ hybridisation with Adenovirus 2 cRNA.

[b] S.A. 1.7 x 10^7cpm/µg; 0.01µg/4µl 2 x SSC; 16 hours at 65°C.

[c] Average of 50 nuclei or metaphase spreads: 8 weeks exposure.

Figure III:5[I]. Hybridisation of Adenovirus 2 cRNA to Adenovirus 2 tumour section(Ad2/T5) *in situ*.a) Tumour was denatured with 0.2N HCl and challenged with Adenovirus 2 cRNA as described in the Legend to Figure III:5O.
Exposure time:8 weeks.
b)Cells in centre of tumour section possess very few grains in comparison to cells over remainder of tumour. Also see Figures IV: 9 and IO of Chapter IV which demonstrate that the centre of Adenovirus tumours are frequently necrotic and little RNA is present.



in transformed cells is originally transcribed from both virus DNA strands (Green and Landgraf-Leurs, 1973; Sambrook et al., 1973; Ozanne et al., 1973; Khoury et al., 1972). Such RNA resembles the completely symmetric transcription of virus-specific sequences in some SV40-infected cells (Aloni, 1972; Fried, 1972) and polyoma-infected mouse kidney cells (Aloni and Locker, 1973).

To test whether Adenovirus tumour cells could have some in vivo RNA which could hybridise to Adenovirus cRNAs, two experiments were performed. First of all, sections of the Ad2/T5 tumour were denatured and then RNased prior to in situ hybridisation with Adenovirus 2 cRNA; secondly, nuclear RNA was prepared and hybridised to Adenovirus 2 cRNA.

In the first experiment grain numbers were reduced in comparison to non-pre-RNased sections: (Table III:16); in the second experiment there is about 10% RNase-resistance in Adenovirus 2 cRNA-Ad2/T5 nuclear RNA combinations but only 3% in Adenovirus 2 cRNA-normal rat cell combinations (Table III:17). Both of these experiments therefore suggest that some of the autoradiographic grains present in some tumours may represent cRNA-in vivo RNA hybrids.

GENERAL CONCLUSIONS

The main conclusions arising from this Chapter, and in particular this Section V, are as follows:

1. Using cRNAs to detect Adenovirus DNA in transformed or tumour cells presents difficulties, stemming mainly from the fact that Adenovirus cRNAs are selectively transcribed. This selective transcription means that only those sequences which are complementary to the major proportion of sequences in the cRNAs will have any

Table III:17

Nuclear RNA (10μg)	[a] cRNA	[b] %RNase-resistance
Ad2/T5	Ad2	9.7
Normal rat liver	Ad2	2.8
Ad2/T5	Ad7	2.6
Normal rat liver	Ad7	2.4

[a] S.A. 1.7×10^7 cpm; 0.01μg in 2 x SSC; 65°C; 16 hours annealing.

[b] % cRNA-cRNA annealing deducted (see Table III:6)

chance of being detected by RNA excess or *in situ* hybridisation. This in turn means that it is difficult to assess the role of virus DNA in transformation or tumourogenesis on the basis of such cRNA-DNA hybridisation experiments. Given this, however, some direct conclusions can be made. These are as follows.

2. By Adenovirus cRNA excess and DNA excess hybridisation two Adenovirus 2 tumours possess around 2-4 copies of virus DNA sequences complementary to the cRNAs. Another Adenovirus 2 tumour, one Adenovirus 12 tumour, and two independently-derived Adenovirus 2 transformed rat cell lines and one Adenovirus 7 transformed hamster cell line possess less than two copies of virus DNA sequences complementary to the Adenovirus cRNAs. In all these cases, of course, virus DNA sequences may also be present which are not complementary to the major proportion of the cRNA sequences.

3. Adenovirus DNA can be detected in single virus-infected cells by *in situ* hybridisation with Adenovirus cRNAs. It cannot, however, be detected in the transformed cell lines studied; neither can it be detected in the Adenovirus 12 tumour or one Adenovirus 2 tumour. Grains present over another Adenovirus 2 tumour after *in situ* hybridisation with Adenovirus 2 cRNA are unlikely to be virus cRNA-virus DNA hybrids: first, DNA sequences complementary to the cRNA are in very few numbers as judged by cRNA excess hybridisation experiments (2-3 copies), and second, there is some evidence for *in vivo* virus RNA-cRNA annealing occurring in this case.

4. Adenovirus DNA in Adenovirus transformed or tumour cells may exist as incomplete genome copies and/or may be integrated into the host DNA. These conclusions are discussed below.

DISCUSSION

1. Use of Adenovirus cRNAs to estimate virus DNA amounts in eukaryote cells

There is little doubt that virus DNA persists in Adenovirus transformed and tumour cells: virus-specific antigens exist (Freeman et al., 1967; Green, 1970; Gallimore, 1974); virus-specific RNA is present in the nucleus (Shimada et al., 1972; Green et al., 1970; Wall et al., 1973; Tsuei et al., 1972) and the cytoplasm (Fujinaga and Green, 1966; 1967; 1968; this Thesis, Chapter IV); and virus-specific sequences are present in DNA isolated from transformed and tumour cells (Green, 1970; Green et al., 1970; Pettersson and Sambrook, 1973). Primarily, however, there is the question of just how much virus DNA there is per cell.

As mentioned previously (see Introduction and Table 1:1) estimates of virus DNA vary according to the method employed and the type of transformed cell or tumour cell. In general, cRNA-DNA hybridisation estimates, as opposed to virus DNA-DNA reassociation estimates, are higher, although this generalisation does not hold good in all cases: the amount of EBV genomes in a variety of human lymphoblastoid cell lines are always large, between 20-100 copies per diploid quantity of host DNA (Zur Hausen et al., 1972; Zur Hausen and Schulte-Holthausen, 1970; Nonoyama and Pagano, 1971; Nonoyama and Pagano, 1973). In the case of "Raji" cells, a line derived from an African Burkitt's lymphoma, 50-52 EBV equivalents per cell were estimated by both virus DNA-DNA reassociation and cRNA-DNA hybridisation.

There are essentially three points which need to be explained. Why, in some cases, do cRNA-DNA hybridisation and virus DNA-DNA

reassociation estimates vary? Second, why, in some cases, do they not vary a great deal and finally, why are estimates obtained by Adenovirus cRNA-DNA hybridisation low as described in this Thesis but high as described elsewhere (see Table 1:1).

One feature of cRNA-DNA hybridisation suggests that this technique might give rise to overestimates.

Virus gene equivalents tend to be estimated in conjunction with reconstruction experiments using pure virus DNA and these experiments, which also use membrane filters, can overestimate the amount of virus DNA sequences present in host DNA because completely homologous hybrids are lost from the filters.. This has been demonstrated by Haas et al. (1972) who showed that SV40 cRNA-SV40 DNA hybrids were not retained during the hybridisation procedure, but SV40 cRNA-transformed cell DNA hybrids were as long as the molecular length of the host DNA exceeded the molecular length of SV40. While this effect might be true for SV40 cRNA-DNA hybrids, it may not be true for other virus cRNA-DNA hybrids however. SV40 cRNA is transcribed asymmetrically from native superhelical double stranded SV40 DNA (Westphal, 1970) and is complementary to all of the genome (Lindstrom and Delbecco, 1972; Khoury and Martin, 1972). Other virus DNAs may be transcribed preferentially, selectively, or symmetrically. Certainly for Adenovirus cRNAs overestimation on the basis of selective loss from filters is unlikely to occur since these cRNAs are preferentially transcribed (Section 11:111). In support of this, estimates on the amount of virus DNA in transformed cells or tumour cells studied here are very low. Three Adenovirus transformed cell lines, for example, contain less than 2 copies of DNA sequences complementary to the

major sequences in the cRNAs. Selective homologous cRNA-DNA hybrid loss from filters may not occur with EBV cRNA-DNA hybrids either since cRNA-DNA hybridisation and virus DNA-DNA reassociation estimates are similar. This point, however, remains to be established.

As pointed out above, overestimation on this basis requires that the transformed or tumour DNA be greater than the molecular length of the cRNA used. In this connection SV40 DNA is approximately 30 times less in molecular weight than EBV DNA: 3×10^6 daltons as opposed to 1×10^8 (Green, 1970; Lindahl and Adams, 1975). Therefore the effect may be more pronounced for SV40 genome equivalent determination than for EBV determination since host DNA isolated for experimentation is usually in the region of 10^7 daltons in molecular weight.

It is less clear why estimates for virus DNA in Adenovirus transformed or tumour DNA should be so different; low as described in this Thesis but reasonably high as described by other workers using cRNA-DNA hybridisation (see Green, 1970; Green *et al.*, 1970).

Many of the experiments in this Thesis have been devoted to showing that Adenovirus cRNAs do not represent a homogeneous population of virus DNA transcripts (Section II:111), a finding which has been corroborated by Pettersson *et al.* (1974); Green and Hodap, (1972); Dunn *et al.*, (1973); and McDougall *et al.*, (1975). And it is clear that this could give rise to underestimates rather than overestimates. Of course under-estimation could occur if the amount of potential hybrid formed was reduced. This situation could arise if, for example, either hybridisation reactions were not taken to saturation or were carried out at supra-optimal temperatures (see

Section III). Decreased saturation, in turn, could be the result of insufficient RNA excess or short, incomplete hybridisation times of incubation. Under-estimation on this basis, however, is extremely unlikely to be a feature of Adenovirus cRNA-DNA estimation at the T.OPT. One is therefore left with the conclusion that either the estimates of Green (1970) and Green et al. (1970) are overestimates due perhaps to a variety of factors, or that the Adenovirus transformed or tumour lines studied by these authors contain more virus DNA than those studied here. Factors which might contribute to overestimation in these cases could be background radioactivity which can be quite high (see Table III:14 for example); overestimation of the analytical complexity of the host rat genome; or overestimation of the amount of transformed DNA immobilised on filters. Green et al. (1970) do use 10^{13} daltons as the value for the analytical complexity of the rat genome (diploid) in contrast to other estimates of 3.6×10^{12} (Steele, 1968) which would therefore lead to an overestimation of around 3 times, bringing their virus DNA estimates to around 3-13 copies per diploid quantity of host DNA. This is partially substantiated by the findings of Pettersson and Sambrook (1973), who using the same Adenovirus 2 transformed cell line (8617) as Green et al. (1970) and Green (1970) demonstrate close to one virus DNA copy per diploid quantity of host DNA, by virus DNA-DNA reassociation (see Table 1:1).

Much the same problem is encountered in estimates of virus DNA sequences by in situ hybridisation. The results presented in this Thesis suggest that no transformed cells studied here possess virus DNA sequences which are detectable at the cytological level. The

same can probably be said for the tumour cells studied since in the tumour which is positive by this technique, cRNA-*in vivo* RNA annealing exists. These findings are consistent with the results obtained by other conventional hybridisation techniques used here:namely, the maximum amount of virus DNA sequences which have been detectable are in the range of 2-4 copies complementary to the cRNAs (see Table III:14, for example). But again, other workers have reported that Adenovirus DNA appears to be detectable at the cytological level. McDougall *et al*. (1972b) and Dunn *et al*. (1973), for example, using Adenovirus cRNA and Adenovirus 12-induced tumours, calculated approximately 50-150 virus genomes per cell on the basis of autoradiographic grain counts after *in situ* hybridisation. They also reported specific detection of Adenovirus 2 DNA in certain Adenovirus transformed cells (Dunn *et al*., 1973). Loni and Green (1973) have also claimed that Adenovirus DNA sequences can be detected by *in situ* hybridisation. Their estimates were 2.7, 10.7, and 5.5 virus DNA copies for Adenovirus 2, 7 and 12 transformed cells respectively. The Adenovirus 12 transformed cell line was the 8617 line (see before). The problems encountered with Adenovirus cRNA hybridisation have already been discussed, and *in situ* hybridisation itself presents limitations on detectibility (Section V; Jones, 1973; Hennig, 1973). Some idea of the feasibility of the *in situ* hybridisation approach in relation to detecting Adenovirus DNA in transformed and tumour cells can be deduced from the present results. The efficiency of the process is around 4-5% (see Section IV) and the reaction is comparable with RNA excess hybridisation. Assuming 2-4 Adenovirus DNA copies per cell, the amount of virus DNA available for detection would be in the range of 6×10^{-11} µg

assuming each cell to have 6 x 10^{-6} µg diploid DNA and the M.W. of Adenovirus DNA to be approximately 20-25 x 10^{6} daltons. The Adenovirus 2 cRNA is synthesised from 5% of the template (see Section II, III) and is complementary mainly to single strand DNA. Thus the amount of complementary virus DNA will be about 6 x 10^{-12} µg per diploid host cell. At a cRNA specific activity of 1.7 x 10^{7} cpm/µg; 10% autoradiographic efficiency and an *in situ* hybridisation efficiency of 4-5%, about 500-1000 days would be needed to obtain 1 grain per cell. These considerations suggest that the *in situ* hybridisation estimates obtained by other workers are over-estimates. This may be due, in part, to the presence of *in vivo* RNA-cRNA hybrids. (Table III:17). There is a precedent for self-complementary RNA in the nucleus of the cell which is absent from the cytoplasm (Aloni, 1972; Aloni and Locker, 1973; Fried, 1972). This would be consistent with the grains restricted to the nuclei of some Adenovirus transformed or tumour cells after *in situ* hybridisation with Adenovirus cRNA as described by McDougall *et al*. (1972b). DNA-DNA reassociation using restriction fragments of the virus DNA as "probes" is a rather more precise method of virus genome estimation. Digestion of Adenovirus 2 DNA with restriction enzyme E COR 1 results in the production of equimolar yields of six DNA fragments, each of which correspond to a unique segment of virus DNA (Pettersson *et al*., 1973). Reassociation of each of these fragments alone, and in the presence of transformed or tumour DNA showed that the Adenovirus 2 transformed cell line, 8617, was missing two complete fragments of the virus genome (Sharp *et al*., 1974). Furthermore, only about 1 copy of each of the other fragments was present, the total percentage of the virus genome present being

46%.

As seen from Table 1:1 (Chapter 1) this line possessed close to one copy by the virus DNA-DNA reassociation technique but up to 30 copies by the cRNA-DNA hybridisation technique. This shows that the cRNA-DNA hybridisation estimates obtained by Green (1970) & Green *et al.* (1970) in particular are overestimates. More importantly it demonstrates that unless the virus DNA sequences are present in the cell they will not be detected by the cRNA.

The finding that an Adenovirus transformed cell line contains incomplete Adenovirus genomes is important. To ascertain whether this lack of specific regions is a general phenomenon of Adenovirus transformation or tumourogenesis, other cell lines were studied (Gallimore *et al.*, 1974). The essential conclusion was that no Adenovirus 2 transformed rat cell line contains DNA sequences homologous to the complete Adenovirus genome, and the same 14% of the lefthand end of the virus genome is always present. The transformed cell line (Ad 2/REB/10p/B1) studied in this Thesis was also studied by Gallimore *et al.* (1974), who demonstrated that it possesses only 5-6 copies of the 14% of the left hand end of the Adenovirus 2 genome. It is also now known that this 14% is not transcribed efficiently *in vitro* by the *E. coli* RNA polymerase (Pettersson *et al.*, 1974). It is therefore hardly surprising that this transformed DNA fails to hybridise either by conventional or *in situ* hybridisation with Adenovirus 2 cRNA as described in this Thesis. Another cell line studied in the present work was derived from the tumour Ad2/5, itself induced by inoculation of Adenovirus 2 transformed rat fibroblast cells without immunosuppression (see Materials). Gallimore *et al.* (1974)

have studied the original transformed cell line - termed T2C4 - and shown that it contains, on average, about 95% of the virus genome per diploid quantity of host DNA. Furthermore, the E CoR I restriction fragments, F and D, are present in about 4 copies. This number is very similar to the 2-3 copies of virus DNA sequences detected by cRNA-DNA hybridisation as described in this Thesis; the D and F fragments likely being the greatest proportion of the cRNA transcript (see discussion before). Subsequent work [Sharp et al., 1974; Cold Spring Harbor Symp. Quant. Biol. 39, 457 (1974.], moreover, has shown that symmetric transcripts of virus DNA sequences exist both in the nucleus and cytoplasm of certain transformed or tumour cells. In particular, and relevant to the present work, is the finding that in the T2C4 line the F fragment, at least, is transcribed equally efficiently from both virus DNA strands. Again, then, the original T2C4 line is similar to the Ad2/5 line described here in that symmetric transcripts have been detected. This means, apart from good correlation and corroboration, that the original line and cells derived from the tumour induced by it possess similar quantities of virus DNA sequences and similar virus RNA expression. This similarity has also been commented on for the transformed BI line (Gallimore et al., 1974) and other transformed and tumour lines as well (Gallimore, personal communication); and it indicates that the amount of virus DNA or its expression in individual cells is unlikely to change through cultivation in vitro or indeed in vivo.

The other cell lines or tumours studied here have not been studied by Gallimore et al. (1974) or others but the finding that most Adenovirus transformed or tumour cell lines contain very few and usually

incomplete virus DNA genomes suggest that this is a general feature of Adenovirus transformation and tumourogenesis.

2. Significance of low levels of virus DNA in Adenovirus transformed or tumour cells

In the present Adenovirus tumour or transformed cell lines studied 2-4 copies of virus DNA represents the maximal amount of virus sequences detected per diploid quantity of DNA. Although the cRNA hybridisation technique would be incapable of detecting certain virus DNA sequences in the cells, by virtue of the fact that the cRNAs are selectively transcribed, it seems likely that 2-4 virus DNA copies is a reasonable estimate of the total number of virus DNA sequences that exist. Adenovirus transformed cells and tumours can contain virus DNA sequences not complementary to the major proportion of sequences in the cRNAs (see above discussion) but in these cases the overall amount per diploid quantity of host DNA is still very low: from a few copies of 14% of the virus genome to only one or two copies of nearly complete genomes. Thus, although it is conceivable that certain cells could contain large numbers of Adenovirus DNA sequences not represented in the virus cRNAs evidence obtained elsewhere indicates that this is unlikely. Many other virus transformed or tumour cell lines including those provoked by SV40, polyoma, and certain Herpes viruses, also contain very low amounts of virus DNA sequences (see Table 1:1; also Cold Spring Harbor Symp. Quant. Biol. 39, 1975). (EBV, however, is an exception in that many copies of its DNA usually exist per transformed or tumour cell). How, then, does this relate to our understanding of transformation or tumourogenesis?

Several points emerge. First, it is striking that although the amount of virus DNA is low per cell, the proportion of virus-specific RNA is usually high (also see Chapter IV): thus suggesting the preferential transcription of virus DNA. Because of this, some of these selectively transcribed DNA sequences are likely to be involved in promoting or maintaining either the transformed or the tumourogenic state. For most transformed or tumour cells the existence of only one gene coding for some transformation or oncogenic function may therefore be enough. Certain other lines of evidence support this view. Graham et al. (1974), for instance, have shown, by naked DNA transformation experiments, that a specific region of the Adenovirus 5 genome amounting to about 6% of the lefthand end appears to be capable of transforming rat kidney cells in culture. Adenovirus 2 transformation or tumourogenesis as well may also be initiated and maintained by the presence of only 14% of the left hand end of the Adenovirus 2 genome (Gallimore et al., 1974). Part of this argument, of course, does imply that both the presence of a single specific gene or set of genes and their transcription does have a direct effect on the initiation or maintenance of transformation or tumourogenesis. Tumourogenesis is likely to involve a whole series of events involving both the expression of the relevant virus gene and the relationship of the virus-exposed cell with the host (e.g. see Klein, 1975b): but there is good evidence, however, which suggests that virus-transformation at least is reliant on the expression of specific, and only a few, maybe one, virus genes. For example, temperature sensitive mutants exist which, in a certain complementation group, fail to initiate or maintain the transformed state at the restrictive temperature. The need for SV40 gene A function in SV40 virus transformation is a case in point (Martin

and Yang Chou, 1975; Brugge and Butel, 1975; Tegtmeyer, 1975; Osborn and Weber, 1975). Even so, the expression of one or two copies of virus-specific genes might not be enough to maintain transformation in certain cases. This may be true for EBV transformation where a characteristic feature of the process is large amounts of EBV DNA per transformed cell. Some cells transformed by EBV even at extremely low m.o.i. still contain multiple copies of virus DNA sequences (Robinson and Miller, 1975) which does suggest that the virus DNA may be subject to amplification which, as a mechanism, could provide the means by which several transformation genes accumulate. The need for this in EBV transformation may be the result of very low levels of virus DNA expression; for example its transcription into RNA or into transformation-specific protein. The fact that, in certain cases, transformation or malignancy can be dependent on a balance or gene dosage of normal and abnormal cell genes also tends to suggest that the amount of virus gene product which affects transformation is likely to be important. For the majority of mammalian DNA viruses this may amount to the expression of one or two copies of the important virus gene: but for EBV, for example, it may mean more.

A second point concerns the role of virus DNA integration. It is unfortunate that the use of <u>in situ</u> hybridisation in studying the chromosomal integration of virus DNA seems not possible. For the majority of virus DNA transformed or tumour cells nevertheless, by CsCl gradient centrifugation of one Adenovirus 2 tumour DNA followed by Adenovirus 2 cRNA hybridisation, virus DNA sequences were detected in the region of the gradient corresponding to the banding of host DNA sequences (see Figures III:42 and III:43). This may indicate that the

virus DNA sequences, in this particular tumour at least, are integrated into the host DNA sequences.

In general the evidence for virus DNA integration into host DNA is reasonably strong (see Chapter 1). Virus DNA integration, *per se*, however, may not be the deciding factor of whether a cell becomes transformed or not. There is now a large amount of evidence which suggests that integration of virus DNA occurs during both productive infection (Burger and Doerfler, 1974; Collins and Sauer, 1972; Hirai and Defendi, 1072; Manor *et al.*, 1973; Waldeck *et al.*, 1973; Ralph and Colter, 1972; Holzel and Sokol, 1974) and abortive infection (Doerfler, 1968; Burlingham and Doerfler, 1971; Doefler, 1970; Doefler *et al.*, 1972) also. However, there are at least two ways in which virus DNA integration appears to differ between productive infection and transformation in particular: the amount of integrated virus DNA is usually more during productive infection, and more importantly there is no definitive evidence to support the hypothesis that integration *per se* contributes functionally to the inevitable replication of the virus DNA, the production of virus particles, and the death of the cell.

More integrated virus DNA during productive infection suggests that there may be several potential integration sites; while the presence of low amounts of virus DNA during transformation suggests that only a selective few of the potential sites are involved in transformation. Other evidence points in this direction: for example, somatic cell hybridisation studies, and the fact that transformation usually occurs with very low frequency. Even for EBV transformation where multiple virus DNA sequences exist per cell it is clear that the potential integration sites must be few; 50 genomes, as

present in "Raji" cells for example, represents about 5×10^9 daltons of DNA amounting to about 1/25th of the DNA in each chromosome which would be a vast amount if it was all integrated.

It is also clear that "transformation" specific sites could exist. There is a precedent for the phenotypic expression of eukaryote genes depending upon their position or orientation in the genome.

Thus there is some case for believing that transformation fundamentally may be the result of the expression of a specific product of a gene residing at a specific site in the eukaryote genome.

CHAPTER IV

ATTEMPTS TO DETECT ADENOVIRUS-SPECIFIC RNA SEQUENCES IN TRANSFORMED AND TUMOUR CELLS

INTRODUCTION

MAMMALIAN DNA VIRUS-SPECIFIC RNA IN TRANSFORMED & TUMOUR CELLS

As mentioned previously virus-specific RNA is found in mammalian DNA virus transformed and tumour cells, for example in Adenovirus transformed and tumour cells (Fujinaga and Green, 1966; 1968; Green, 1970); polyoma transformed cells (Banjamin, 1966); SV40 transformed cells (Aloni et al., 1968; Oda and Dulbecco, 1968; Reich et al., 1966; Sauer and Kidwai, 1968); and EBV transformed human lymphoblastoid cell lines (Sudgen, personal communication). In some transformed or tumour cells it represents a considerable percentage of the RNA in the cell whereas in others the percentage is less so. Adenovirus transformed cells, for example, possess 2-5% virus-specific polysomal messenger (m) RNA (Green, 1970), whereas most SV40 or polyoma transformed cells possess only about 0.01-0.1%.

In general, cells transformed by mammalian DNA viruses usually possess only a subset of the virus-specific RNA sequences found in productive infection (Green, 1970; Green et al., 1970; Botchan et al., 1974; Sambrook et al., 1972; Khoury et al., 1974; Sharp et al., 1975).

Three different classes of virus mRNA molecules are synthesised in Adenovirus transformed or tumour cells, each one being specific for each major sub-group of the human Adenoviruses (Fujinaga et al., 1969; McAllister et al., 1969; Green, 1970). And in the main, only 4-10% of the Adenovirus genome is expressed, the sequences being a subset of the sequences expressed early in the productive cycle.

(Green and Hodap, 1972). In other transformed cells there is also selective transcription of the virus genome. Thus some SV40 transformed cells express only about one third of the virus genome (Aloni et al., 1968) while others express less or sometimes more. Most SV40 transformed cells, however, do differ from Adenovirus transformed cells studied in that some late virus genes are transcribed in the former but not in the latter. In some cases this late gene expression can amount to nearly 80% of the normally expressed late genes in productive infection (Sauer and Kidwai, 1968).

For SV40 transformed 3T3 cells virus-specific RNA is complementary to 55-60% of the sequences of the early strand of the SV40 DNA molecule, and 15-20% of the sequences of the late strand (Sambrook et al., 1972). For some other SV40 transformed cells (Ozanne et al., 1973), 30-80% of the early strand and 0-20% of the late strand are present as RNA. Since the early region in production infection corresponds to about 30-35% of the early strand (Lindstrom and Dulbecco, 1972; Khoury et al., 1972; Sambrook et al., 1972; Sambrook et al., 1973), a proportion of the RNA produced in certain SV40 transformed cells is clearly anti-late. Two main points emerge from this data. First, there is selective transcription of virus genes in transformed or tumour cells. Second, this selectivity may be the result of control mechanisms or loss of virus DNA sequences. In SV40 transformed cells late genes can be transcribed, while in Adenovirus transformed cells they are not. This may reflect the fact that all the virus genome is present in most SV40 transformed cells but not in Adenovirus transformed cells (see pg. 4).

VIRUS-SPECIFIC RNA AND VIRUS DNA INTEGRATION

Virus-specific nuclear RNA in transformed or tumour cells appears to be covalently-linked to cellular HnRNA (Heterogeneous nuclear RNA). Thus virus DNA - either SV40 or Adenovirus for example - hybridises to nuclear transformed RNA sequences greater than 40-45S in sucrose gradients with or without DMSO treatment to disrupt aggregation (Linberg and Darnell, 1970; Green, 1970; Green et al., 1970; Wall et al., 1973). Similar experiments, using polyacrylamide gels, have shown that SV40 or Adenovirus-specific transformed cell nuclear RNA sequences exist in high molecular weight form (Young et al., 1973; Green et al., 1970). Since infectious virus DNA is not normally found in these cells, and the amount of virus DNA present probably represents only a few genome copies (see Chapter III, this Thesis and also Gelb et al., 1971; Botchan et al., 1974; Sharp et al., 1974; Gallimore et al., 1974) it is unlikely (but not impossible) that the high molecular weight virus-specific nuclear RNA is due to tandemly repeated, or continuous, transcription of the virus DNA only.

A proportion of the high molecular weight RNA could possibly be due to the post-transcriptional addition of ribonucleotides. PolyA, for instance, is added to most of the eukaryote primary RNA transcripts which give rise to mRNA (Lewin, 1974; 1975) and to DNA virus-specific RNA in cells (e.g. Philipson et al., 1971). However, since some virus-specific mRNA, in transformed cells, still exceeds the length of the expected virus DNA sequence transcript, (e.g. Weinberg et al., 1973) even allowing for 50,000 daltons of polyA, the influence of polyA on the size of virus-specific sequences in HnRNA must be small.

Virus-host HnRNA molecules also can exist in cells productively-infected by several mammalian DNA viruses (Tonegawa et al., 1970; Acheson et al., 1971; Green et al., 1970; Weinberg et al., 1972; Jaenisch, 1972; Rozenblatt and Winocour, 1972).

Tsuei et al. (1972) and Wall and Darnell (1972) isolated the virus-host HnRNA in Adenovirus and SV40 transformed cells. RNA sequences which wereco-isolated with pure virus-specific sequences hybridised to cell DNA under contitions which suggest that these sequences are transcribed from repetitive DNA. Whether any of the host HnRNA sequences which are linked to virus-specific sequences are transcribed from unique DNA sequences in the genome or whether any virus-specific RNA sequences in the nucleus are not transcriptionally linked to host HnRNA sequences is not known.

The difficulty with these sort of experiments is the unresolved question of whether or not RNA aggregation can occur to any great extent, thus bringing about high-molecular weight RNA. Macnaughton et al. (1974), for example, have shown that distinct mRNA species can aggregate and that RNA-RNA interactions can occur in nuclear RNA when full RNA denaturation conditions are omitted. This may also be true for virus-specific RNA - host HnRNA interactions, particularly when non-denaturation conditions are employed. Judged from this point of view, integration of virus DNA within the host genome may be artifactual. The virus-specific RNA is subsequently transported to the cytoplasm through a series of post-transcriptional modifications and cleavage (Shimada et al., 1972; Wall and Darnell, 1971; Wall et al., 1973) which results in discrete classes of virus-specific messenger RNA (mRNA). For example, three discrete size classes of virus-specific

mRNA have been found for one Adenovirus 2 transformed cell line: 16S, 20S and 26S (Wall et al., 1973). More heterogeneous size classes have been found for other Adenovirus transformed cells (Green et al., 1970).

DESIRABILITY OF DETECTING VIRUS-SPECIFIC RNA IN INDIVIDUAL CELLS

The discovery that virus DNA can exist in several types of transformed or tumour cells has lead to many attempts to try and detect it in different types of tumours. However this may not be an easy task since, in many cases, the amount of virus DNA may be relatively low (see Chapter III, this Thesis). Screening tumours by in situ hybridisation with virus cRNAs (McAllister et al., 1972; McDougall et al., 1972b; Wolf et al., 1973; Chapter III, this Thesis) may not therefore be the method of choice. RNA transcription, however, represents direct amplification of the virus DNA sequences - even at a selective level. Consequently a method was devised by which virus-specific RNA could be detected in individual cells. The particular usefulness of the method is that virus-specific nucleic acids can be detected in cells in which the virus DNA content would be too low to detect. This raises the possibility of screening for virus-specific sequences in cancer tissue in which the virus DNA, if present, is limited to a few genome copies.

A. DETECTION OF ADENOVIRUS-SPECIFIC MESSENGER RNA(mRNA) IN INDIVIDUAL TRANSFORMED CELLS

SECTION I

Preliminary isolation of Adenovirus 2 messenger RNA from Adenovirus 2 transformed rat cells

^{3}H-Uridine-labelled virus-specific mRNA was isolated from Adenovirus 2 transformed cells (Ad 2/REB/10p/B1) by isolating total mRNA via polyA-mRNA-oligo(dT) binding and hybridising it to virus DNA immobilised on filters. The results show that virus-specific mRNA can be detected in these transformed cells.

Results and discussion

a) Shimada et al. (1972) have shown that in certain Adenovirus transformed cells virus-specific RNA can be detected in the nucleus after 30 mins. incorporation of ^{3}H-uridine. After 60 mins. there is also a high proportion of virus-specific RNA in the cytoplasmic RNA. Further, the proportion of virus-specific RNA in the nucleus compared to the cytoplasm decreases by 95% in 4 hours of incorporation time. After several hours of labelling with ^{3}H-Uridine the virus-specific messenger RNA (mRNA) has dramatically accumulated in the cytoplasm (Tsuei et al., 1972). In order to check that the transformed cells could incorporate ^{3}H-Uridine under the conditions employed, BI transformed cells were labelled with ^{3}H-Uridine for various time periods and the radioactivity determined by TCA precipitation of whole cells (Figure IV:1). Incorporation is linear with a slight falling off after 60-90 mins. BI transformed cells were labelled for up to 8 hours before RNA was extracted.

Figure IV:I. Incorporation of ^{3}H-Uridine into total cellular RNA with time. Adenovirus 2 transformed rat cells (Ad2/REB/IOp/BI) were labelled with IOuCi/ml ^{3}H-Uridine(S.A. 25Ci/mmole) for various times.Whole cells were then washed with changes of Dulbecco A and finally TCA precipitated onto GF/80 filters. After alcohol drying and heating at 80^{o}C for 40mins.,the radioactivity on the filters was determined by counting in Toluene-POPOP,PPO scintillation fluid.

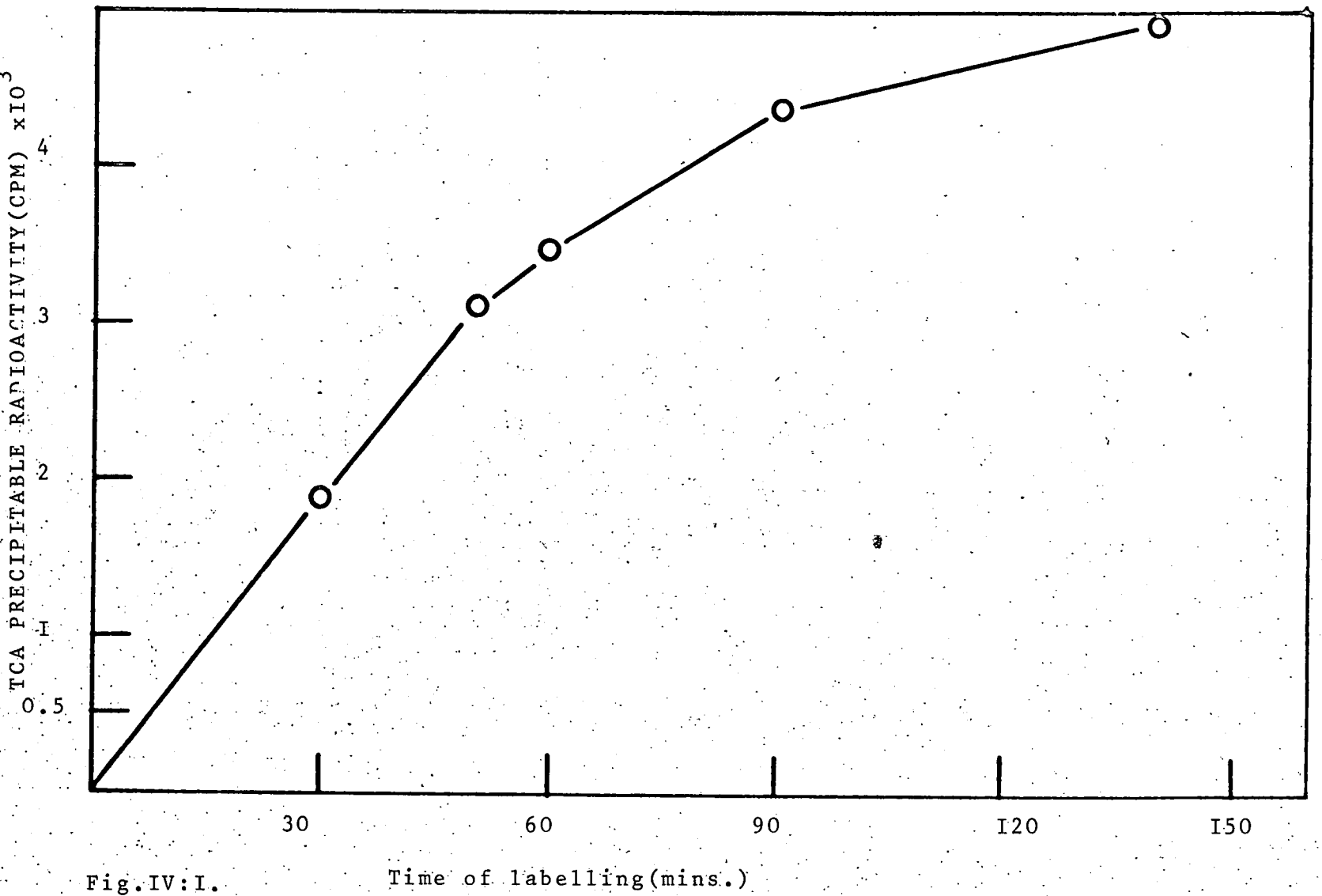

TCA PRECIPITABLE RADIOACTIVITY (CPM) xIO3
4
3
2
1
0.5
30
60
90
I20
I50
Time of labelling (mins.)

Fig.IV:I.

b) Cytoplasmic RNA was extracted and purified by a combination of methods. (see Materials and Methods, pg. 56). This RNA was passed through an oligo(dT) column and the polyA-containing RNA eluted in low salt (Aviv and Leder, 1972). Approximately 5% of the ^{3}H-Uridine-labelled RNA was eluted in low salt after two passages through the column (Figure IV:2).

c) The polyA-mRNA was hybridised to Adenovirus 2 DNA and M. luteus DNA which were both immobilised on membrane filters. The specific activity of the ^{3}H-Uridine-labelled mRNA was 2×10^{5}cpm/µg. Hybridisation was performed in 6 x SSC 30% FA at 50°C and the reaction terminated by chilling, RNasing and washing the filters. The result is shown in Table IV:1. The mRNA hybridises to the virus DNA but not to the bacterial DNA. Table IV:1 also shows that polyA-mRNA isolated from normal rat cells does not significantly bind to the Adenovirus 2 DNA. All this demonstrates that the polyA-mRNA isolated from these particular Adenovirus 2 transformed cells contains Adenovirus 2-specific sequences. From these experiments, the proportion of the polyA-mRNA which is virus-specific cannot be dtermined. Neither can the virus DNA sequence complementarity be determined. Nonetheless, virus-specific mRNA clearly exists and should be capable of being detected by in situ hybridisation.

Figure IV:2. Separation of polyA-mRNA from total cytoplasmic RNA isolated from Adenovirus 2 transformed rat cells(Ad2/REB/IOp/BI).The polyA-mRNA was eluted in low salt buffer (ImM EDTA,IOmM Tris-O.I% SLS)(arrow) and the radioactivity determined by counting in liquid scintillator (Aquasol).

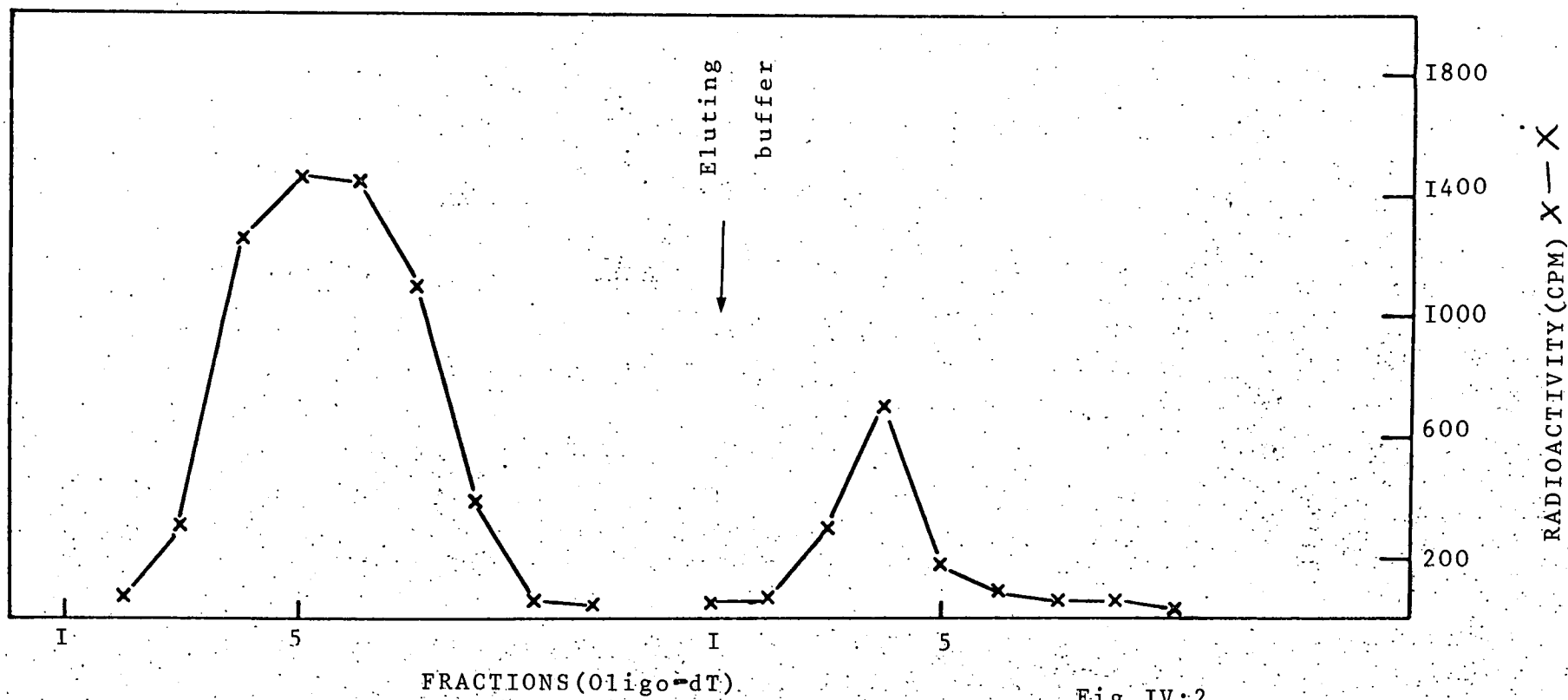

Eluting buffer
1800
1400
1000
600
200
RADIOACTIVITY(CPM) X — X
I
5
I
5
FRACTIONS(Oligo-dT)

Fig.IV:2.

Table IV:1

DNA	^{3}H-Uridine-labelled Adenovirus 2$_5$ transformed cell RNA (S.A. 2 x 10^5 cpm)	c.p.m. hybridized[a,b]
Adenovirus 2 (5µg)	4 x 10^5	820
M. luteus (5µg)	4 x 10^5	55
M. luteus (20µg)	4 x 10^5	67
DNA	^{3}H-Uridine-labelled normal rat fibroblast cell RNA (S.A. 2 x 10^5)	c.p.m. hybridized[a,b]
Adenovirus 2 (5µg)	4.3 x 10^5	60
M. luteus (5µg)	4.0 x 10^5	43

[a] Average of 2 experiments

[b] Hybridisation at 50°C in 6 x SSC 30% FA for 10 hours

SECTION II

Detection of mRNA in individual cells by in situ hybridisation

Specific RNA sequences have been detected in individual cells before. Harrison et al. (1973), for instance, localised Haemoglobin messenger RNA in the cytoplasm of red blood cells using in situ hybridisation with Haemoglobin cDNA. Detecting specific RNA sequences at the single cell level is therefore feasible.

The experiments described here were designed to examine three points. Can Adenovirus-specific RNA sequences be detected at the single cell level and if so, is the technique specific and reliable. Third, does specific virus messenger expression in individual cells vary between cells? The first two points involve hybridisation of ^{3}H-labelled Adenovirus 2 DNA to transformed and infected cells. The third point also exploits the formation of hybrid complexes between polyA-mRNA and polyU to estimate total mRNA expression in comparison to virus-specific mRNA expression.

Results and discussion

Adenovirus 2 DNA labelled with ^{3}H-Thymidine during the growth cycle in HEK cells, and purified as described in Materials and Methods possesses a buoyant density in neutral CsCl of 1.716gm/cm^{-3} (Figure IV:3), a value in good agreement with previously reported density determinations for this particular virus DNA (Table III:1, this Thesis; Green and Pina, 1964; Ledinko and Fong, 1969). There is no apparent label in the density position of human main band DNA, a result which suggests the purity of the virus DNA. When the virus DNA is denatured it is virtually completely S1 nuclease sensitive, and on reassociation it becomes 50% S1 nuclease resistant when a

Figure IV:3. CsCl gradient centrifugation of ^{3}H-Thymidine labelled Adenovirus 2 DNA.The labelled DNA in O.IxSSC,together with total human DNA(unlabelled) and M.luteus DNA(unlabelled) was made up with 5.$_{2}$gm CsCl(B.D.H. analytically pure) to a volume of 4.Omls.Centrifugation was done at 42,000 rpm at 25^{o}C for 40hours in the IOxIO MSE rotor. Fractions were collected,their O.D.s (260nm) determined,and the DNA in each fraction denatured,neutralised,and bound to membrane filters according to the procedure of Gillespie and Spiegelman(I965).The radioactivity of each fraction was then determined by counting the filters in Toluene-based scintillation fluid after the DNA had been washed in 6xSSC and baked at 80^{o}C for 2hours in vacuo. The peak of radioactivity corresponds to the buoyant density of Adenovirus 2 DNA in neutral CsCl(see TableIII:2); while M.luteus DNA and total human DNAs also band at their known buoyant density positions of I.73Igm/cm^{-3}and I.700gm/cm^{-3} respectively.(also see TableIII:I).

OPTICAL DENSITY(260nm)
0.1
0.2
0.3
Rotor Centre
I.731
I.700
1
10
20
FRACTIONS
1.0
2.0
3.0
RADIOACTIVITY(CPMxIO4).

Fig.IV:3.

$Cot\frac{1}{2}$ of approximately 1.7×10^{-2} moles/sec./l^{-1} has been reached (Figure IV:4). The rate and extent of the reassociating virus DNA is similar to that expected for a unique genome with an analytical complexity of around 25×10^{6} daltons which is the molecular weight of Adenovirus 2 DNA (Green et al., 1967 for example). Other workers have observed similar $Cot\frac{1}{2}$ values for this particular virus DNA (Tibbetts et al., 1974; Pettersson et al., 1974). Thus by buoyant density measurement and by reassociation kinetics the labelled DNA represents a homogeneous population of Adenovirus genomes. This virus DNA was hybridised to Adenovirus 2 transformed rat cells (Ad 2/REB/10p/B1). These cells are heavily labelled in the cytoplasm with occasional grain clusters in the nucleus (Figure IV:5b, 5c). There were few grains in either the nucleus or the cytoplasm of normal rat cells. To test whether label represents RNA-DNA hybrid formation, Adenovirus 2 DNA was hybridised to transformed cells which had been previously RNAsed (20 µg/ml, RNase A in 2 x SSC for 30 mins.) and exhaustively rinsed in 2 x SSC. After such treatment the amount of label was considerably reduced subsequent to in situ hybridisation (Figure IV:5d). The relatively low labelling in the nucleus of a transformed cell compared to the cytoplasm (Figure IV:5) may reflect the apparently lower content of virus-specific sequences in HnRNA in comparison to their proportion in the mRNA of the polysomes (Shimada et al., 1972). Alternatively, the efficiency of hybridisation to the nucleus might be impaired for some reason. To test whether the Adenovirus 2 DNA was capable of hybridising to virus sequences in the nucleus of a cell, an in situ hybridisation reaction was carried out with the labelled virus DNA and HEK cells infected with Adenovirus 2.

Figure IV:4. Reassociation of ^{3}H-Thymidine labelled Adenovirus 2 DNA. 3xSSC, 65°C, with an initial DNA concentration of $3xIO^{-I}$ ug/ml. % reassociation was measured by SI nuclease monitoring(see Materials and Methods, pg. 57).

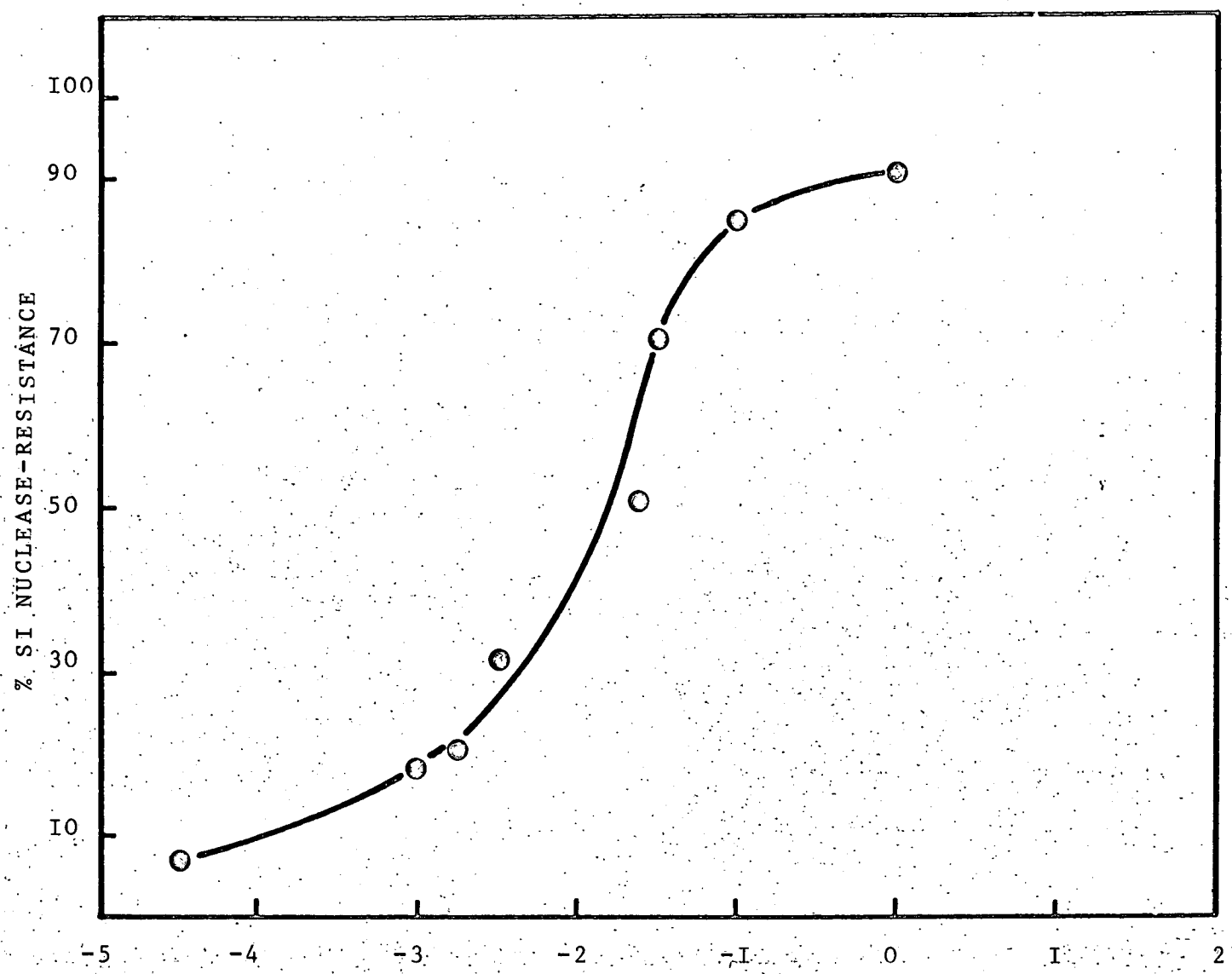


Fig.IV:4.

Cells permissive for Adenovirus infection are known to possess viral-specific RNA both in the nucleus and the cytoplasm (Green et al., 1970; Green, 1970; Fujinaga et al., 1968), and to accumulate newly-synthesised viral DNA in the nucleus (Green, 1962; Ledinko and Fong, 1969). Furthermore, newly-replicated viral DNA can be detected by in situ hybridisation with virus cRNA (McDougall et al., 1972a; Gallimore, 1974; this Thesis pg.122) so that Adenovirus 2 replicated duplexes, syntheised in the infected HEK cells, should be capable of forming duplexes with the ^{3}H-labelled Adenovirus 2 DNA used to detect virus sequences in the transformed cells. Hybridisation to pre-RNAsed or non pre-RNAsed Adenovirus 2-infected HEK cells or nuclei is depicted in Figures IV:5a and IV:6b. Grains are located in the nuclei of pre-RNAsed cells (Figure IV:6b) and in both the nuclei and cytoplasm of non pre-RNAsed infected cells (Figure IV:5a). A pre-RNAsed Adenovirus 2-infected HEK nucleus which has been in situ hybridised with Adenovirus 2 cRNA is shown in Figure IV:6a for comparison. These above results suggest that the input Adenovirus 2 DNA can hybridise or anneal to either virus-specific RNA or virus-specific DNA in the Adenovirus 2-infected cells. There were few grains in either the nucleus or cytoplasm of normal rat cells.

During the course of the reaction, the virus DNA would be expected to reassociate or self-anneal, as well as hybridise to any complementary base-sequences within the cell. Other studies have shown that this self-renaturation of DNA sequences probably does not present problems.

For example, Pardue and Gall (1969) detected amplified ribosomal DNA in situ by employing double stranded labelled ribosomal DNA. The centromeric location of mouse satellite DNA has also been demonstrated

Figure IV:6. In situ hybridisation of Adenovirus cRNA or Adenovirus 2 DNA to Adenovirus-infected cells. Adenovirus 2or I2-infected HEK cells(IOOpfu/cll) were pre-RNased and challenged with either Adenovirus 2cRNA or I2cRNA (S.A. I.7xIO^{7}cpm/ug;0.OOIug/5ul; 65^{o}C for IOhours in 2xSSC) or Adenovirus 2 DNA(S.A. IO^{6}cpm/ug: IO^{-3}ug/ul:65^{o}C for IOhours in 3xSSC).
a) Adenovirus 2 cRNA hybridised to Adenovirus 2-infected HEK cells;
b) Adenovirus 2 DNA annealed to Adenovirus-2-infected HEK nuclei;
c) Adenovirus I2 cRNA hybridised to Adenovirus I2-infected HEK nuclei;
d) Adenovirus 2 DNA annealed to Adenovirus I2-infected HEK nuclei.
Exposure time:4-6 weeks.

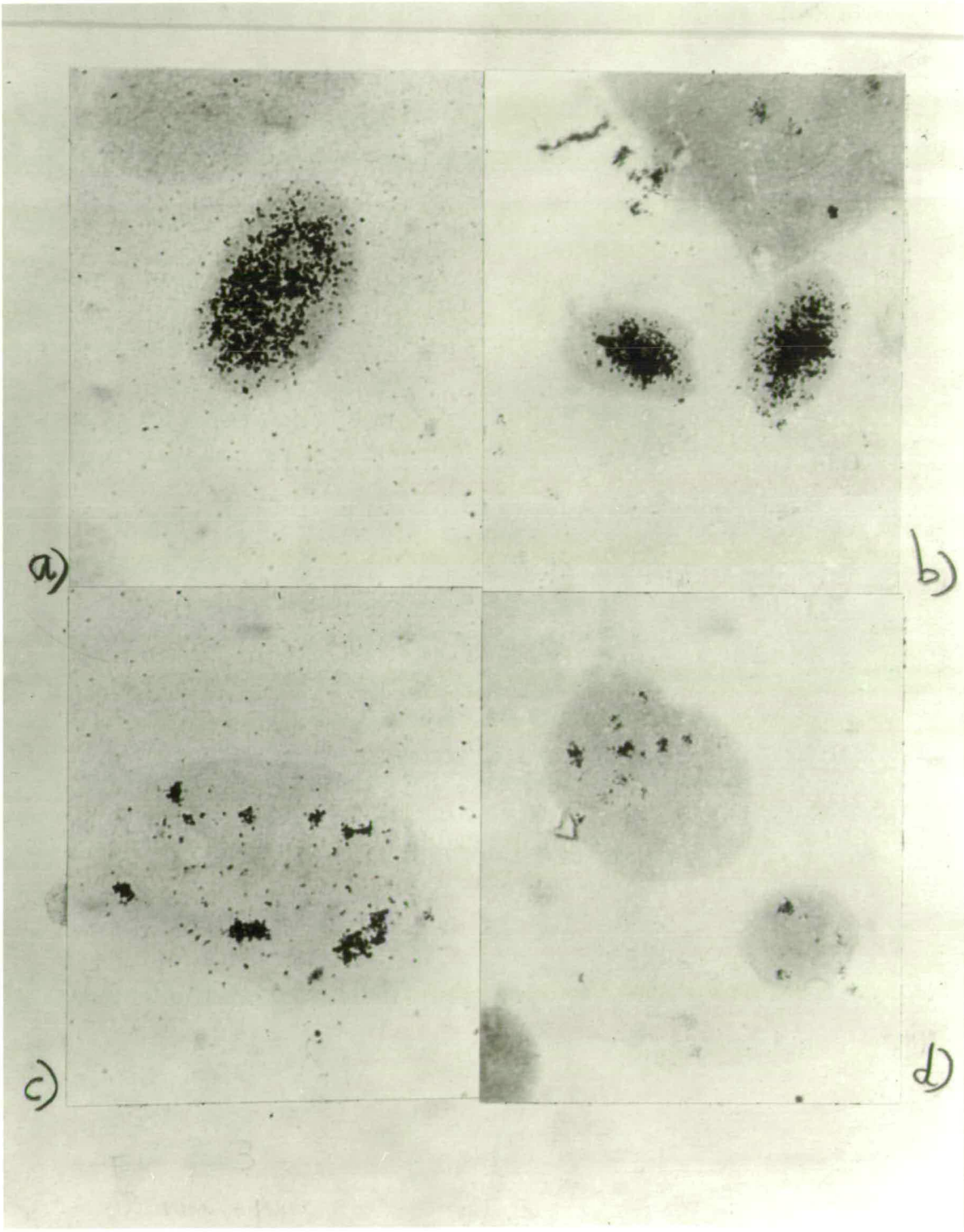
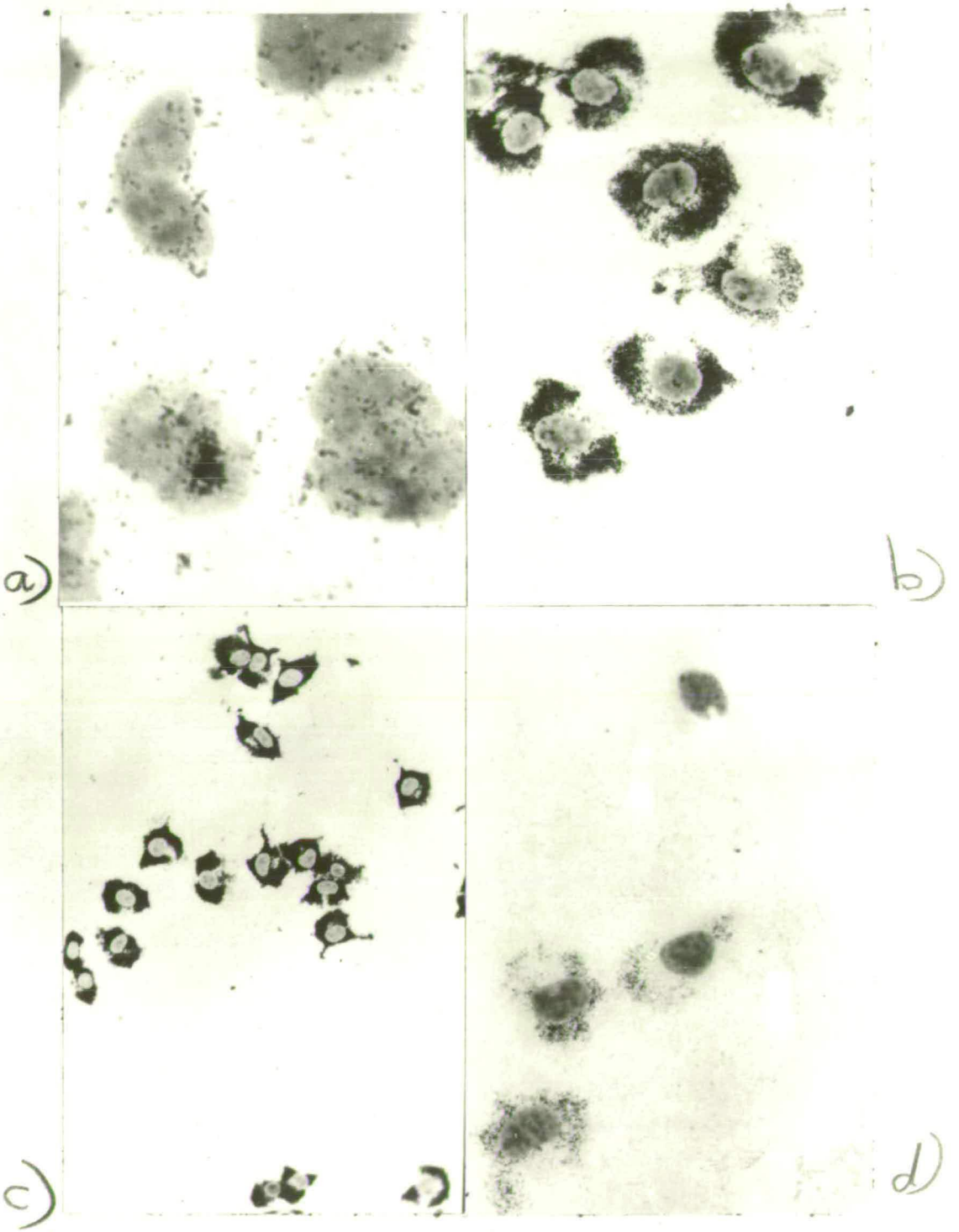

a)
b)
c)
d)

Figure IV:5. In situ hybridisation of Adenovirus 2 DNA to Adenovirus 2 -infected HEK cells or to Adenovirus 2 transformed rat cells.
a) Adenovirus 2 DNA annealed to Adenovirus 2-infected HEK cells: no pre-RNasing.
B) and c) Adenovirus 2DNA hybridised to Adenovirus 2 transformed rat cells with no pre-RNasing: $3xIO^{-3}$ug/ml: 3xSSC 30%FA: 45°C for IOhours(3xSSC for IOhours at 65°C also gave same results).
d) Adenovirus 2 DNA hybridised to Adenovirus 2 transformed rat cells pre-RNased.Conditions of hybridisation as for b).
Exposure time: a)4weeks;b),c) and d) 6 months.


a)
b)
c)
d)

using mouse satellite DNA as a probe (Pardue and Gall, 1970) and recently, Hennen et al. (1975) have located the position of the ribosomal genes on the mitotic and lampbrush chromosomes of Triturus cristatus carnifex using I-125-labelled double stranded ribosomal DNA. The ability to do this is fortunate since self-annealed DNA base-sequences could contribute to the autoradiographic label because they will be S1 nuclease resistant, we well as compete with the hybridisation reaction at the cytological level.

The Adenovirus 2 DNA, at the criteria used here, would be expected to have nearly half-reassociated within an hour of the commencement of the reaction in situ (Figure IV;4). However there is no extracellular background radioactivity and hybridisation is very much greater in transformed cells than in normal rat cells. The grains present in large amounts in the cytoplasm of these transformed rat cells most likely represent virus in vivo RNA- virus DNA hybrids since RNAsing the cells prior to in situ hybridisation drastically reduces the label. There is some evidence from other in situ hybridisation experiments that pre-RNasing may not remove all the hybridisable RNA (Hennen et al., 1975; Rosbash, personal communication) so that the remaining grains, after such treatment, possibly represent some non-RNased molecules which have hybridised. To further check the specificity of the in situ reaction an additional two experiments were performed. First, the Adenovirus 2 labelled DNA was hybridised to Adenovirus 12 infected cells which contain replicating virus DNA (Green, 1970; McDougall et al., 1972) detectable by in situ hybridisation with Adenovirus 12 cRNA (Section III, Chapter III; Figure IV:6c also). Adenovirus 12 and 2 DNAs share up to 22% homology. Therefore

Adenovirus 2 labelled DNA should be capable of hybridising to Adenovirus 12 DNA in these cells. Figure IV:6d shows that this is so. Grain clusters are present in individual nuclei. The second experiment involved hybridising a non-virus labelled DNA to cells which do not contain RNA sequences complementary to it. Transformed cells were hybridised with a ^{3}H-Thymidine-labelled Drosophila satellite DNA,(a gift from M. Izquierdo, this laboratory).

The level of grains present after annealing with this satellite DNA was very low (not shown) thus confirming that annealing with ^{3}H-Thymidine-labelled Adenovirus 2 DNA is most probably specific.

Silver grains seen in some control preparations may be the result of either incomplete enzyme digestion or insufficient post *in situ* hybridisation washing. If some of these grains are due to non-specific binding of double-stranded ^{3}H-DNA then the use of separated virus DNA strands may be an advantage in future exploitation of the technique.

The finding that virus-specific mRNA can be detected by *in situ* hybridisation with virus DNA raises some interesting points. First, as described previously, there have been some attempts, using cRNA to Adenovirus genomes, to detect viral-specific DNA in individual cells transformed by Adenoviruses (Green *et al.*, 1970; Dunn *et al.*, 1973; McDougall *et al.*, 1972b; Loni and Green, 1973; Chapter III, this Thesis). The major limitation with this approach, however, is set by the amount of DNA base-sequences which can participate in the reaction, and be subsequently detected by the cRNA in the *in situ* technique. To date in the Adenovirus transformed cells studied, only a portion of the viral genome is present (Sharp *et al.*, 1974;

Gallimore et al., 1974) and in very few copies (Pettersson and Sambrook, 1973; Gallimore et al., 1974; this Thesis, Chapter III), thus limiting the chance of detecting viral DNA. In fact since a large percentage of the cRNA represents only a proportion of the Adenovirus genome (Green and Hodap, 1972; Dunn et al., 1973; Pettersson et al., 1974; this Thesis, Chapter III) screening transformed cells and tumours for virus DNA by hybridisation with virus cRNA (McAllister et al., 1972; McDougall et al., 1972b; Dunn et al., 1973) is unlikely to be a method of choice. As demonstrated here, however, viral-specific RNA can be localised in cells expressing Adenovirus genetic information. Two points in particular suggest that this technique is very sensitive. First, the particular transformed cell line used in this study contains only 14% of the Adenovirus 2 genome (Gallimore et al., 1974) and expresses only approximately 7% of this in the form of a single transcript (Sharp et al., Cold Spring Harbor Symp. Quant. Biol. (1975) in press). Second, Adenovirus 2 DNA is capable of annealing to Adenovirus 12 DNA in infected cells at the cytological level (Figure IV:6d) and yet these two viruses only share 9-22% of their DNA base-sequences (Green et al., 1970). Thus the input Adenovirus DNA is capable of annealing with nucleic acid sequences in the range of 1-2 x 10^6 daltons in molecular weight, i.e. 3-6 x 10^3 base-pairs in molecular length. The ability to detect virus-specific RNA sequences in individual cells should therefore have important uses in general: for screening purposes, and for studying the transcriptional specificities of individual cells in a tumour. For instance, some cells in a certain tumour possess different genetic information from other cells in the same tumour

(Goldenberg et al., 1974; Weiner et al.,1972) and at least one tumour has a restricted distribution of virus genes within its cellular architecture (Wolf et al., 1973; Klein et al., 1974). Little is known about the transcriptional specificities at the single cell level and the method described here therefore has important applications in this direction.

A second point can be raised from the ability to detect specific RNA sequences in situ. It is unclear in general whether mRNA varies quantitatively from cell to cell: for instance during different stages of the cell cycle. Figure IV:5 shows that the expression of virus-specific RNA in Adenovirus 2 transformed cells does not appear to vary much between cells. Whether uniformity in this RNA expression reflects uniformity in total mRNA expression is not clear. An approach to answer this question can be made by exploiting hybrid formation between polyA-mRNA and polyU, the extent of the reaction being a measure of the total mRNA content of the cell. The presence of polyA sequences on mRNA molecules has been alluded to previously. With the exception of Histone mRNAs (Adesnik and Darnell, 1972) mRNA sequences contain polyA post-transcriptionally added to the 3' end of the messenger sequences thus enabling the mRNA sequences to be isolated from the rest of the cellular RNA. The amount of polyA in total RNA populations is therefore a measure of the amount of polyA-mRNA sequences. PolyA-mRNA molecules can form complexes with polyU (Bishop et al., 1974; Rosbach et al., 1974) which are then a measure of the amount of polyA-mRNA in an RNA population (see Rosbash and Ford; 1974 for example).

To determine whether total mRNA expression varies from cell to cell, as opposed to virus-specific mRNA expression which does not appear to (see Figure IV:5), polyU was hybridised to transformed cells (see below).

PolyU-homopolymer complexes are less stable than a variety of other RNA-DNA hybrids (Riley et al., 1966; Chamberlin, 1965), and low temperatures of RNasing these hybrids are needed in comparison to other RNA-DNA hybrids (e.g. Bishop et al., 1974). PolyU-poly(rA) complexes are more stable than polyU-poly(dA) complexes. For instance, polyU-poly(rA) has a Tm of 72°C in 2 x SSC whereas polyU-poly(dA) has a Tm, in the same salt, of 42°C. This Tm difference is demonstrated in Figure IV:7 where ^{3}H-polyU has been hybridised to poly(rA) or poly(dA) sequences in Drosophila DNA, and the resulting complexes melted in 2 x SSC, Bishop et al. (1974) have shown that mRNA-polyU complexes melt with Tms characteristic of polyU-poly(rA) complexes i.e. around 70°C in 2 x SSC. Figure IV:7 shows in common with Shenkin and Burdon (1974) and Bishop et al. (1974), that at around 50°C the polyU-DNA sequences have approximately 90% melted whereas polyU-poly(rA) complexes are still stable. This is a useful finding since hybridisation carried out at around 50°C in 2 x SSC will largely reflect the polyU-poly(rA) or polyU-mRNA complex formation. In situ hybridisation using polyU was therefore carried out according to the method of Jones, Bishop and Brito-da-Cunha (1973) but with the modification of increased temperature of hybridisation in 2 x SSC: i.e. 50°C instead of 30-37°C.

PolyU hybridisation to transformed cells is shown in Figure IV:8. Transformed cells are heavily labelled, especially in the cytoplasm

Figure IV:7. Thermal dissociation of polyU-DNA or polyU-poly rA complexes.The complexes were melted in 2xSSC and the %polyU released determined by TCA precipitation and counting in Toluene-based scintillation fluid.The Tm is the temperature at which 50% of the polyU-DNA or polyU-polyrA complexes have dissociated.
o-o polyU-drosophila DNA;
O_O polyU-polyrA(Shenkin and Burdon,I974);
x-x polyU-poly rA.

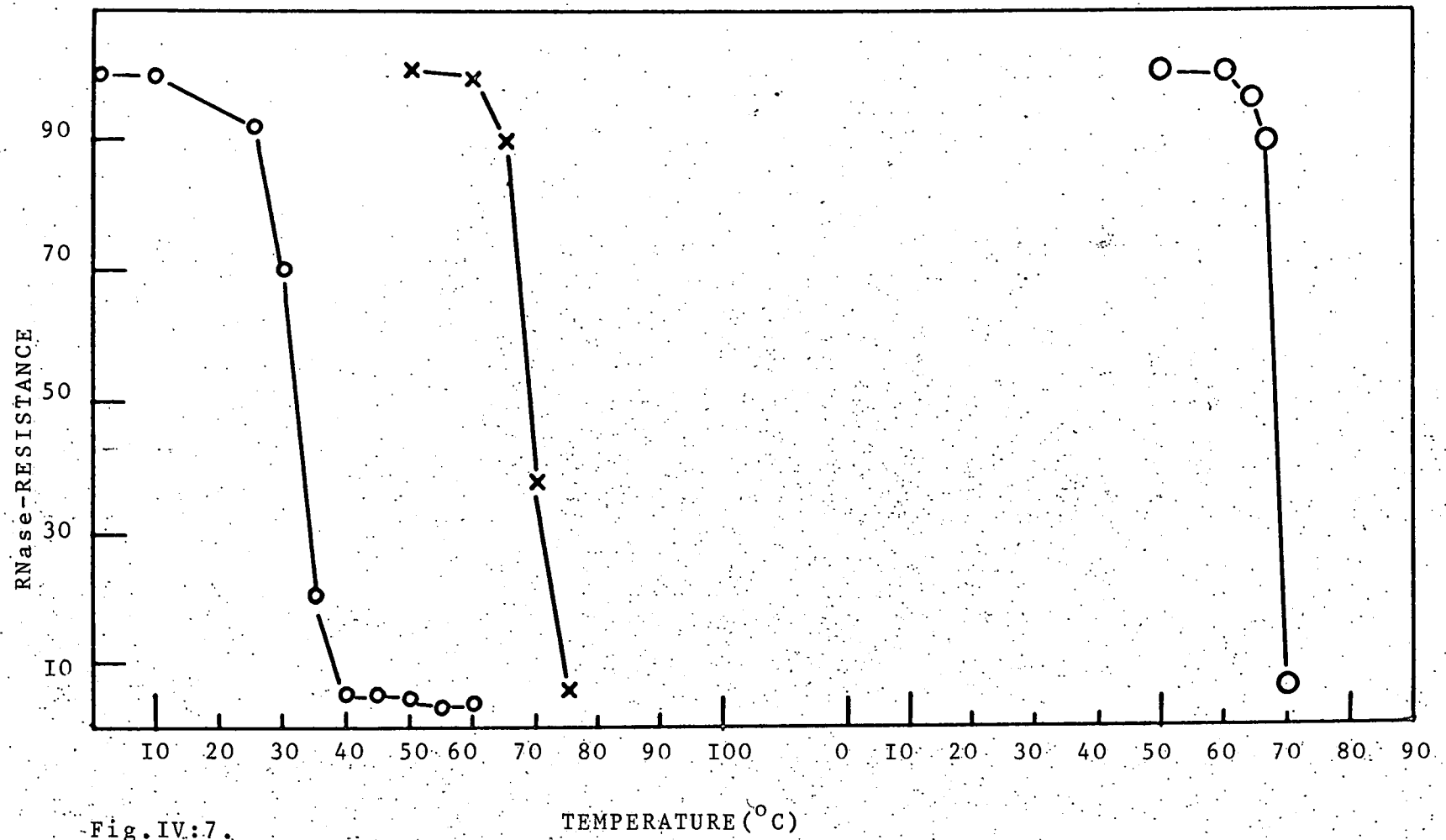
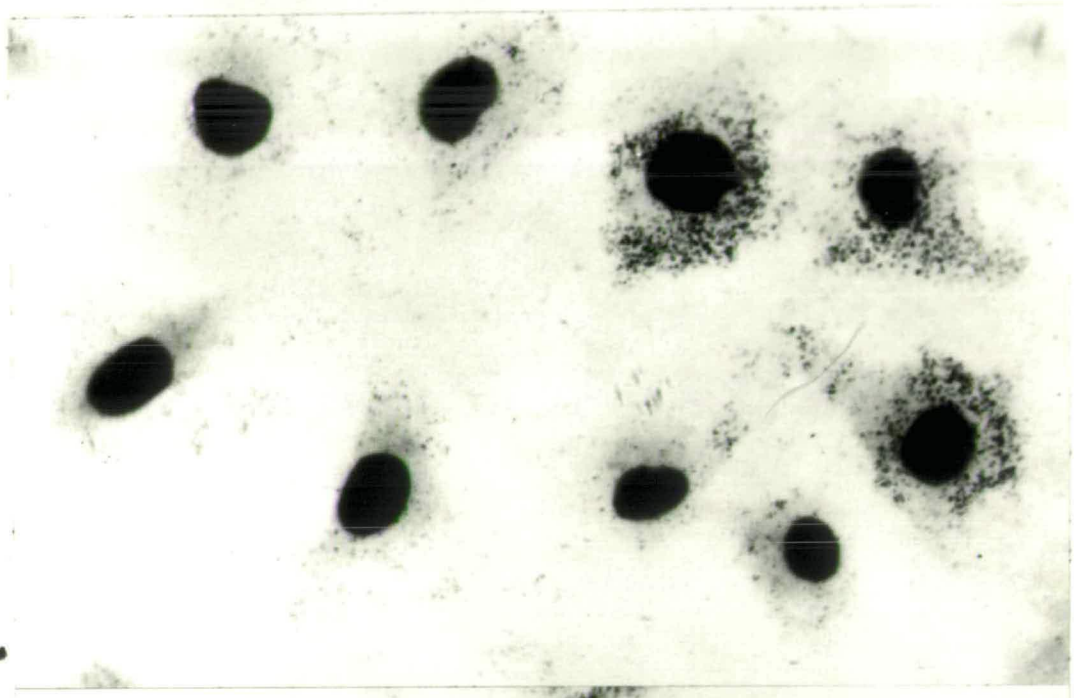
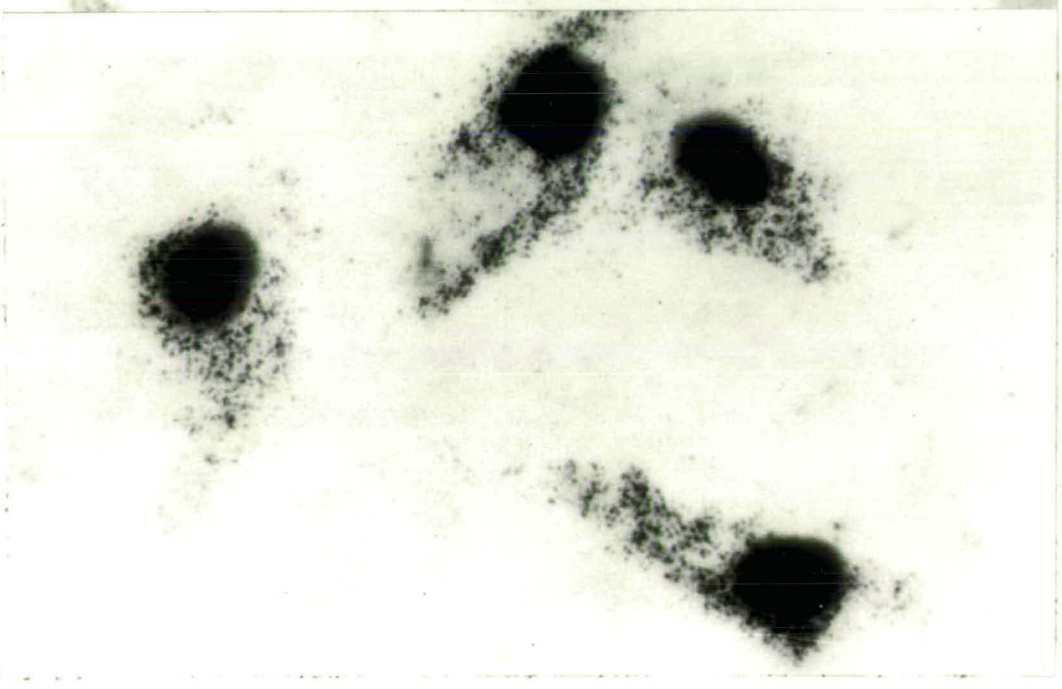

RNase-RESISTANCE
90
70
50
30
10
10 20 30 40 50 60 70 80 90 100
0 10 20 30 40 50 60 70 80 90
TEMPERATURE (°C)

Fig.IV:7.

Figure IV:8. *In situ* hybridisation of poly U to Adenovirus 2 transformed rat cells(Ad2/REB/IOp/BI).Label is concentrated in the cytoplasm.(nuclei are darkly stained with Giemsa).Exposure time:2 weeks.See Materials and Methods and text for details.



a.



b.

where the polyA-mRNA is likely to be accumulating. There is little difference between the labelling pattern observed for several cells. which therefore suggests that the uniformity in Adenovirus-specific mRNA (Figure IV:5) probably reflects the uniformity of polyA-mRNA expression in general.

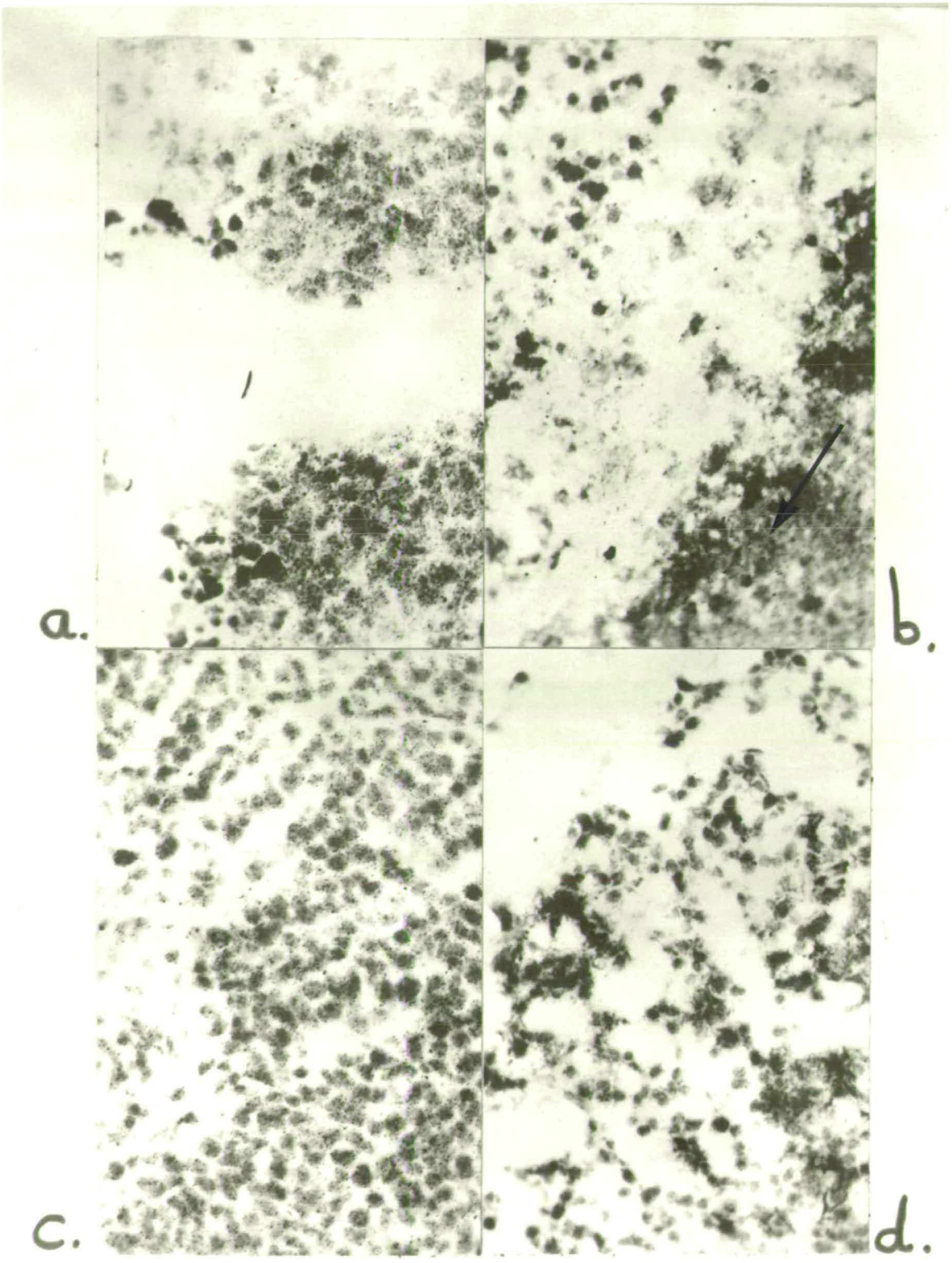
B. TRANSCRIPTION OF RNA IN TUMOURS

The biology of whole tumours is not understood in depth. Reflecting this, while many of the studies on tumour expression have concentrated on analysing tumour cells derived from whole tumours and grown in culture, few studies have directly analysed the tumour as originally produced *in vivo*. Thus many studies have been directed at detecting virus RNA in individual tumour cells isolated from whole tumours but few have attempted to relate this to the biology of the tumour mass (see for example, Axelrod *et al.*, 1964; Green, 1970). As previously demonstrated in this Thesis (pg.157) mRNA expression can be quantitated by polyU binding to individual cells. This method of determining mRNA amount was therefore applied to whole tumour sections in order to determine whether there was heterogeneous total mRNA expression throughout the structure of the tumour mass. The results represent the first report of mRNA estimation for individual cells within a tumour.

Results and Discussion

^{3}H-polyU was hybridised to Adenovirus 2 transformed cell-induced tumour sections or to Adenovirus 12-induced tumour sections. The conditions of hybridisation and synthesis of polyU have already been described (see page 157; and Materials and Methods, pg.59-60). In the Adenovirus 2 transformed cell induced tumour, (Ad2/T5) most of the label is confined to the peripheral cells of the tumour mass (Figures IV:9a & 9b). In the other tumour (Ad12/TI) there is more widespread distribution of label except for cells in the centre of the tumour which appear to possess little (Figures IV:9c and 9d). Thus for the tumour sections there is not uniformity of polyA-mRNA expression. The simplest interpretation of this result is that polyA-mRNA content is diminished in certain cells of the tumour, an interpretation which

Figure IV:9. In situ hybridisation of poly U to frozen sections of Adenovirus-induced tumours.a)and b) Adenovirus 2 transformed cell(Ad2/REB/50p/BI) -induced tumour(Ad2/T_5).Note hybridisation to cells in periphery of tumour mass. b) Adenovirus I2-induced tumour(AdI2/TI). Note widespread distribution of grains except for cells in the centre of the tumour(d) where grains are absent. These centre cells are most likely necrotic.For conditions of polyU hybridisation see Materials amd Methods and text.Exposure time:all 2 weeks.


a.
b.
c.
d.

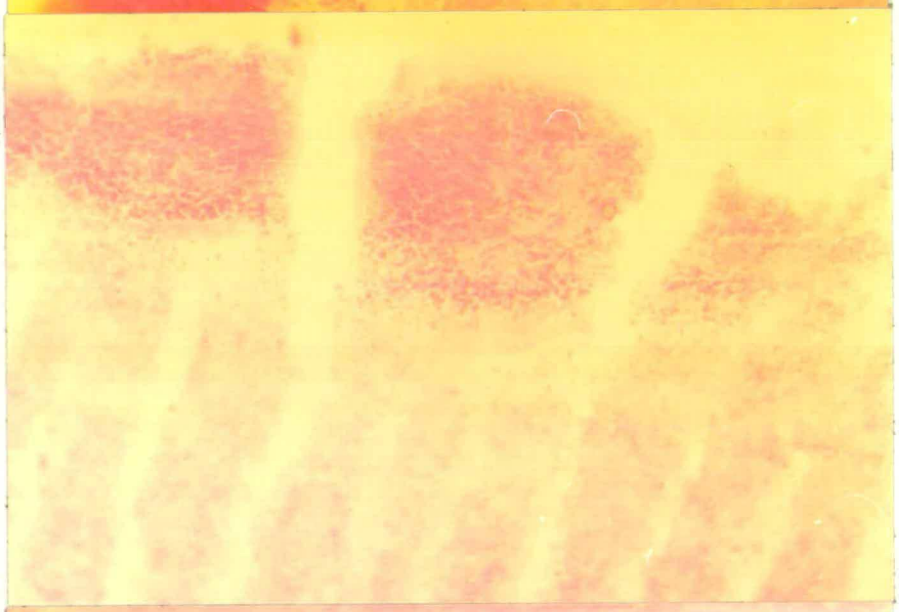
gains support from the fact that many Adenovirus-induced tumours possess necrotic cells which are frequently a major part of the tumour; live cells being only confined to the growing surface. Thus the intense polyU labelling seen in the periphery of one tumour in particular (Figure IV:9a & 9b) probably reflects such a situation. This is further strengthened by the fact that, on staining with Methyl Green Pyronin (MGP) to discriminate RNA from DNA, only the peripheral cells of this particular tumour appear to contain RNA (Figure IV:10a & b). The tumour which contains a more widespread distribution of polyU also contains a more widespread distribution of RNA on staining with M.G.P. (Figure IV:10c). Therefore the polyU bound by cells does reflect *in vivo* RNA expression.

Clearly it will be important and interesting to determine whether there is a similar distribution of virus-specific RNA within these types of tumour. The method described previously should be capable of answering this question.

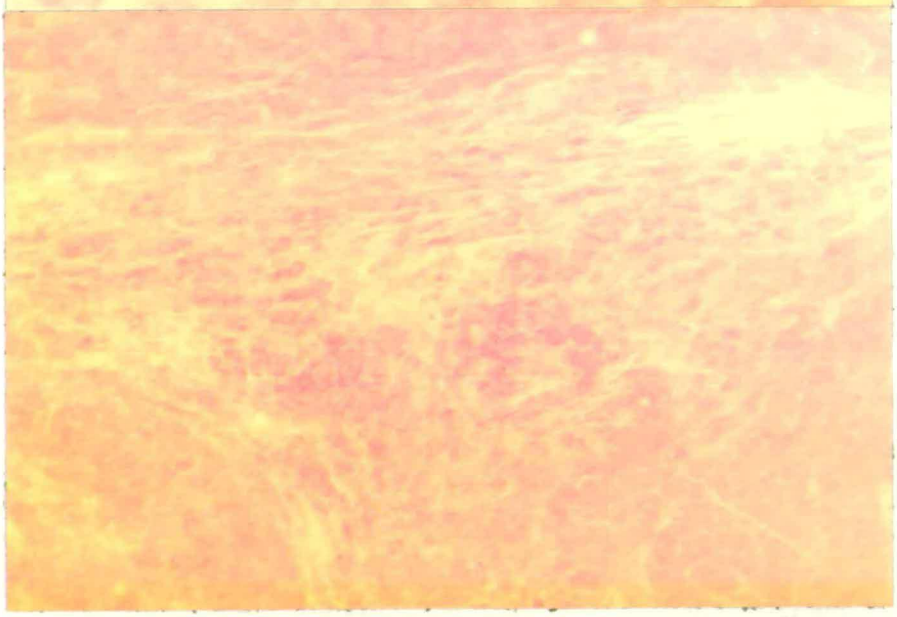
Figure IV:IO. Tumour sections stained with Methyl Green Pyronin(M.G.P.) to discriminate RNA from DNA.(RNA stains distinctly red).
a) and b) Adenovirus 2 tumour section (Ad2/T5):RNA is concentrated in the peripheral cells of the tumour mass.
c) Adenovirus I2 tumour(AdI2/TI): there is a widespread distribution of RNA.

a)

b)



c)



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