THE UNIVERSITY OF MANITOBA

ISOLATION AND CHARACTERIZATION OF UNUSUAL POLYPHOSPHORYLATED DINUCLEOSIDES (HS) IN EUKARYOTIC CELLS

BY

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ABSTRACT

Three unusual dinucleotides given the pseudonym HS (hot spot) 3, 2 and 1 (LéJohn et al. 1975) have been isolated and purified from the fungus Achlya and from Chinese hamster ovary (CHO) cells grown in culture (HS3 only). They have proved to be extremely complex dinucleoside-like molecules.

HS3 from Achlya has been shown to contain adenosine, uridine, D-glutamate, mannitol, a sugar having the characteristics of an amino-polyol, and 8 phosphates per molecule. A structural analysis using chemical (HCl and KOH hydrolysis), physical (ultraviolet light spectral analysis and ion exchange chromatography) and enzymatic digestion methods on HS3, showed it to be a moiety of ADP-sugar X-glutamate* that is covalently linked by a carboxyphospho anhydride group to a UDP-mannitol tetraphosphate moiety. HS3 isolated from CHO cells grown in culture was shown to be chromatographically identical to Achlya HS3 on DEAE-Sephadex at pH 8 and 3.6, and on PEI-cellulose in two dimensions.

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*modified glutamate

Mammalian HS3 (from CHO cells) displayed similar spectral characteristics as Achlya HS3 and mammalian HS3 was found to contain adenosine, uridine, glutamate and 8 phosphates per molecule. On the basis of these data it is concluded that mammalian HS3 is probably identical to that isolated from Achlya.

Only the general chemical compositions of HS2 and HS1 were determined. HS2 was found to contain two uridines, ribose, mannitol and 9-10 phosphates per molecule. HS1 also contains two uridines, ribose and 12 phosphates per molecule. The possibility that both HS2 and HS1 contain the UDP-mannitol tetraphosphate moiety found in HS3 is discussed.

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ABBREVIATIONS

ADP	-adenosine 5'-diphosphate
AMP	-adenosine 5'-monophosphate
AppppA	-diadenosine 5', 5'-tetraphosphate
ATP	-adenosine 5'-triphosphate
cAMP	-cyclic 3', 5'-adenosine monophosphate
cGMP	-cyclic 3', 5'-guanosine monophosphate
CHO	-Chinese hamster ovary
CoA-s-s-CoA	-oxidized coenzyme A dimer
CTP	-cytidine 5'-triphosphate
cUMP	-cyclic 3',5'-uridine monophosphate
EDTA	-ethylenediamine tetraacetic acid
glu	-glutamate
GTP	-guanosine 5'-triphosphate
HCl	-hydrochloric acid
HS	-hot spot
nm	-nanometers
O.D.	-optical density
PEI	-polyethyleneimine
Pi	-inorganic phosphate
POPOP	-1,4-Bis-(2(5-phenyl oxazolyl))-benzene
ppApp	-adenosine 3'diphosphate, 5'diphosphate

pppApp	-adenosine 3'triphosphate, 5'diphosphate
pppAppp	-adenosine 3'triphosphate, 5'triphosphate
ppGpp	-guanosine 3'diphosphate, 5'diphosphate
pppGpp	-guanosine 3'triphosphate, 5'diphosphate
PPO	-2,5-diphenyl oxazole
ppZpUp	-uridine 3'phosphate, 5'phospho-Z-diphosphate
PS	-phantom spot
TCA	-trichloroacetic acid
TEAB	-triethylammonium bicarbonate
tris	-tris (hydroxy methyl) amino methane
UDP	-uridine 5'diphosphate
UMP	-uridine 5'monophosphate
3'UMP	-uridine 3'monophosphate
2'UMP	-uridine 2'monophosphate
urd	-uridine
UTP	-uridine triphosphate
uv	-ultraviolet

INTRODUCTION

Most organisms can respond quickly to subtle changes in the nutritional status of their environment. For example, fungi respond to a depletion of certain essential nutrients such as nitrogen or phosphate by producing spores. The more sophisticated animal cells respond by altering their metabolic usage of the limited nutrient(s). Basically, growth control is affected. It is therefore of great interest to investigators to understand the mechanisms by which the cell can'sense'these nutritional deficiencies and regulate its metabolism to allow continued viability. This is particularily important for the 'deranged' tumor cell.

In bacteria, deficiency in the availability of amino acids for growth can result in the cellular accumulation of guanosine tetraphosphate (ppGpp) which is synthesized on ribosomes because of an idling in protein synthesis. This unusual nucleotide is postulated as a key regulator of various biosynthetic events such as inhibition of RNA synthesis (the phenomenon termed the 'stringent response').

The ppGpp system has not been demonstrated in eukaryotes; however, relevant investigations have led to the discovery of other highly phosphorylated nucleotides some of which have been structurally characterized. The subject of this thesis is a description of the results of a characterization of one of

the many uncommon polyphosphorylated nucleotides now found in eukaryotes. This nucleotide is one of three that have been found in many fungal cells and is also produced in mammalian cells. It has been named HS3 and the other two (only in fungal cells) are HS2 and HS1. Both fungal and mammalian cell HS compounds were studied. Initially these compounds were tentatively identified as diguanosine polyphosphates (LeJohn et al 1975) but have now been recharacterized and found to be much more complex and not guanosine-containing at all. The results show that HS3 is a composite of adenosine, uridine, glutamate, aminopolyol sugar, mannitol and 8 phosphates. Data is also presented suggesting the possible molecular organization of these components in the intact molecule of HS3.

HS2 and 1, present only in fungi, are also complex. They are polyphosphorylated dinucleotides of uracil containing 10 and 12 phosphates respectively. Not much is known as yet about their structures.

Some of the physiology and metabolism of HS3 such as its appearance during ontogeny of the mold, supression of its production by glutamine and correspondingly a block in sporulation suggested that it could be involved in regulating the metabolism of the fungal or mammalian cell. For this and other reasons that will be exemplified in the text, it became vital to structurally characterize the molecule.

The HS compounds are potent inhibitors of RNA polymerases (McNaughton et al 1975), ribonucleotide reductases(Lewis et al 1977)

and DNA polymerases (HS1 only) (LeJohn et al 1978) suggesting that they may play a vital role in regulating nucleic acid metabolism in eukaryotes. Concordant physiological studies have lent support to this concept.

The knowledge gained so far about the chemistry of these HS compounds in conjunction with physiological and genetic studies (of both fungal and mammalian cells) are persuasive enough for one to contemplate that they could play a role in the overall regulation of nucleic acid metabolism (hence growth) in eukaryotes. They could do this by mediating both the transport of necessary pre cursors and their biosynthesis into nucleic acids.

This thesis presents the data obtained on the chemical characterization of the HS compounds, particularily of HS3. A review of the physiological and genetic findings of HS compounds is presented in the 'Historical' section together with a discussion of the properties of other unusual nucleotides found in both prokaryotes and eukaryotes. The rationale behind this approach is to see if there is an underlying metabolic theme for these nucleotidic compounds as possible cellular regulators.

HISTORICAL

HISTORICAL

UNUSUAL NUCLEOTIDES

In recent years much effort has gone into research involving unusual nucleotides and their possible role in mediating specific cellular control mechanisms. The cursory review of the literature which follows shows that a variety of these unusual compounds have been found in both prokaryotes and eukaryotes.

In Prokaryotes

A number of unusual nucleotides have been shown to exist in bacterial cells. These include cyclic AMP, cyclic GMP, cyclic UMP, MS (magic spot) nucleotides, Phantom nucleotide, and the HPN (highly phosphorylated nucleotide) compounds. Cyclic AMP has been shown to regulate a number of metabolic processes in bacteria, including for example its role in catabolite repression of several inducible

enzyme systems (Pastan and Pearlman, 1972).

The MS nucleotides ppGpp (MSI) and pppGpp (MSII) have been found in a variety of bacteria (Gallant and Lazzarini, 1976) and are known to accumulate under amino acid deficient conditions with resultant decline in overall RNA biosynthesis - a phenomenon termed the "stringent response" (Cashel 1970). MSI is believed to be the intracellular regulator involved in this stringent response.

Another nucleotide found to accumulate in E.coli in response to carbon source shiftdown has been reported by Gallant et al. (1976). This molecule was given the pseudonym "phantom spot" (PS), and is postulated to be a modified guanine nucleotide. No specific role has been assigned to it.

Rhaese et al. (1972, 1975a) found four nucleotides in the differentiating prokaryote Bacillus subtilis. These compounds were given the pseudonyms HPN (highly phosphorylated nucleotide) I, II, III, and IV. The tentative structures of these compounds that were reported are as follows: HPNI, ppApp; HPNII, pppApp; HPNIII, ppZpUp (Z is an undefined sugar); and HPNIV, pppAppp. The cellular levels

(based primarily on phosphate labelling kinetics) of these compounds were found to increase in Bacillus in a concomitant manner with sporulation or during carbon or phosphate depletion. Bacillus subtilis was also purported to produce MSI and MSII (Rhaese et al. 1975b).

In Eukaryotes

Cyclic AMP and the HPN nucleotides which were found in prokaryotes were also reported to be produced in eukaryotes. Other unusual nucleotides known to exist in eukaryotes are cyclic GMP, HS (hot spot) 1, 2, 3, N^6-substituted adenines and diadenosine tetraphosphate (AppppA)

Cyclic AMP seems to play a regulatory role in both prokaryotes and eukaryotes. In the slime molds, cyclic AMP is considered to be involved in the aggregation and differentiation process (Konijn 1972). Cyclic AMP has also been reported to be present in yeast where it is thought to play a role similiar to its function in catabolite repression in bacteria (Van Wijk and Konijn 1971).

In higher eukaryotes (mammalian cells) cyclic AMP is an intracellular mediator of several hormone-induced responses (Sutherland 1972). Cyclic AMP has also been implicated as a negative effector in the regulation of cell growth and proliferation (Pastan 1975).

Cyclic GMP has also been found in both prokaryotic and eukaryotic organisms (Goldberg and Haddox 1977). Studies on mammalian cell tissues showed that agents or conditions which antagonized cyclic AMP accumulation facilitated an increase in the cellular levels of cyclic GMP. This has led to the suggestion that these two molecules promote opposing processes in the regulation of cell growth and proliferation (Pastan 1975).

Three unusual nucleotides given the pseudonyms HS (hot spot) 1, 2 and 3 have been reported to occur in a variety of fungi (LéJohn et al. 1975). These compounds have recently been characterized as dinucleoside polyphosphates (McNaughton et al. 1978). They too have been implicated in the regulation of cell growth and sporulation in the water mold Achlya (LéJohn et al. 1978). In addition, Goh and LéJohn (1977) showed that HS3 was present in

mammalian cells in culture. This compound was found to accumulate when glutamine was removed from the growth medium or when some vital growth nutriment was unavailable. Mammalian HS3 was shown to be identical chemically and physically to HS3 isolated from Achlya (Lewis et al. 1977).

Rapaport and Zamecnik (1976) reported that they had detected the existence of yet another unusual nucleotide diadenosine tetraphosphate (AppppA), in cultured mammalian cells and animal tissues. The cellular levels of this compound were reported to decrease when serum was removed from the growth medium. This is the opposite effect to HS compounds.

Rhaese et al. (1975b) reported that the HPN nucleotides also existed in various mammalian cell lines in culture. These compounds were shown to accumulate when an inhibitor of DNA and RNA synthesis was added to the growth medium.

All these nucleotides described above are considered unusual because, although they contain all the constituents of common ribonucleotides (nitrogen base, ribose and phosphate (s)), they differ with respect to the number of phosphates, type of phosphate bonding, the presence of other

components (sugars, amino acids), or the number of nitrogen bases (dinucleotide structures). Generally these nucleotides do not contain modifications to the nitrogen base(s), however, there is one possible exception. It has been suggested that the compound known as "Phantom Spot" might contain such a modification.

A common feature of these unusual nucleotides is that they are all considered to have a cellular regulatory function, that is, mediation of a specific cellular response to an environmental stress. As will become apparent later, the end result of such a stress to the cell may be an increased or decreased growth rate, a rapid change in the metabolic program of the cell, or in the process of sporulation (differentiation).

A wealth of information has already been gleaned from experimental study on two of these unusual nucleotides, ppGpp and cyclic AMP, with respect to cellular control mechanisms mediated by them in prokaryotes (ppGpp and cyclic AMP) and eukaryotes (cAMP). The results of these investigations led Tomkins (1975) to describe a generalised

model of cellular control in eukaryotes in which analogous regulatory mechanisms were correlated into a complex regulatory processes.

COMPLEX REGULATION

In 1975 Tomkins proposed a model to describe possible control mechanisms by which cells could alter their metabolic processes in response to changes in their environment. Tomkins suggested that cellular regulatory mechanisms could be classified into two groups. The first, designated simple regulatory processes are exemplified by substrates or end products which affect their own metabolism. The essential feature of simple processes is that there is a direct chemical relationship between the regulatory molecules and their effects. Simple regulation may be positive as in enzyme induction or negative as in feedback inhibition of enzyme activity and repression of enzyme biosynthesis.

Tomkins termed the second group complex regulatory processes which he defined as two entities, "metabolic symbols" and their "domains", which do not operate by simple mechanisms. A metabolic symbol is defined as a specific intracellular effector molecule which accumulates when the cell is exposed to a particular environment. Two examples considered as metabolic symbols were cyclic AMP and ppGpp. The domain of a symbol is defined as

all the metabolic processes over which the symbol exerts control. Tomkins points out that the various biochemical reactions included in the domain of a symbol need only be related by their biological effects rather than chemical mechanisms.

Metabolic symbols are signal molecules, their intracellular accumulation indicating a change in the environment of the cell. The regulation of specific metabolic processes (domain) allows the cell to adapt to the new environment.

Tomkins suggested that a complex regulator (metabolic symbol) should be a relatively small molecule and labile to allow its concentration to fluctuate quickly. He also suggested that the symbol or enzyme(s) synthesizing them should be in close association with the cell membrane.

The concepts described in this model were based largely on data gleaned from studies on cyclic AMP and ppGpp. However, Tomkins suggested that other regulatory processes might operate under the same principles, and be mediated by small molecules such as ppGpp and cyclic AMP.

As has already been discussed in the previous section a number of unusual nucleotides have been

discovered since this model was published. These studies are probably a direct result of the work done on cyclic AMP and ppGpp as well as Tomkins' model. Whether these newly-found compounds can be classed as "metabolic symbols" is still questionable as data concerning most of those described are still scant. Most of the reports (except for cyclic AMP and ppGpp) have appeared in the last three years.

The following sections will attempt to describe some properties of these unusual nucleotides.

THE MS NUCLEOTIDES: MEDIATORS OF STRINGENT CONTROL

The Stringent Response

The observation that bacterial cells show a reduction in the rate at which RNA accumulates when protein synthesis is slowed down by the removal of amino acid was made many years ago (Sands and Roberts 1952). This general response was then found to result in a complete cessation of RNA accumulation when auxotrophic strains of E.coli were deprived of an essential amino acid (Pardee and Prestidge 1956). This suggested that bacterial cells possessed a mechanism which could regulate RNA synthesis with regard to its ability to make protein. This phenomenon was termed stringent or amino acid control (Stent and Brenner 1961).

Measurements of RNA synthesis and degradation suggested that this restriction of accumulation was most probably at the level of synthesis (Travers 1974). Studies on the type(s) of RNA synthesis that was being restricted suggested that virtually all stable RNA (rRNA and tRNA) accumulation ceased upon amino acid starvation but mRNA synthesis was

only partially inhibited.

Other metabolic processes are also inhibited during the stringent response. These include carbohydrate synthesis (Sokawa et al. 1970); lipid and phospholipid synthesis (Golden and Powel 1972; Merly and Pizer 1973; Sokawa et al. 1968, 1970); de novo nucleotide synthesis (Gallant et al. 1971); peptidoglycan synthesis (Ishiguro and Ramey 1977); membrane transport of nucleobases (Hochstadt-Ozer and Cashel 1972) and glycosides (Sokawa and Kaziro 1968). Also observed was an increased rate in proteolysis (Sussman and Gilvarg 1969). These results indicated that the stringent control mechanism was not limited to RNA accumulation but control was probably exerted on a number of biosynthetic pathways resulting in a complete reorganization of the cell's metabolic program.

The Stringent Factor: Synthesis of Two Unusual Nucleotides

Genetic studies with relaxed (rel^-) mutant strains which do not exhibit the stringent phenotype and stringent (rel^+) strains ascribed the stringent

phenotype to a single genetic locus designated the RC gene (Stent and Brenner 1961; Alfoldi et al. 1962; Lavelle 1962; Fiil and Friesen 1968). Fiil (1969) showed that rel^- is recessive to rel^+ suggesting that the rel gene product, termed stringent factor, acts directly or indirectly to inhibit RNA accumulation.

A search for a possible mediator in the stringent response led to the discovery of two unusual nucleotides, designated MSI and MSII, which were found to accumulate at the onset of amino acid starvation in stringent strains but not in relaxed strains of E.coli (Cashel and Gallant 1969). These two unusual nucleotides were characterized as guanosine 3'diphosphate 5'diphosphate (MSI) and guanosine 3'diphosphate 5'triphosphate (MSII) (Cashel and Kalabacher 1969; Sy and Lipman 1973; Que et al. 1973). These compounds have also been shown to accumulate under similiar conditions in other bacteria (Swanton and Edlin 1972; Gallant and Margason 1972; Rhaese et al. 1975).

The formation of these two molecules is believed to be stimulated by an 'idling' reaction of protein synthesis caused by the presence of an uncharged tRNA molecule on the acceptor site of the ribosome (Cashel and Gallant 1969; Cashel 1969; Hazeltine et al.

1972; Hazeltine and Block 1973). The enzyme which synthesizes MSI and MSII (stringent factor) is thought to be the product of the RC gene (Lazzarini et al. 1971; Harshman and Yamazaki 1971, 1972; Lund and Kjeldgaard 1972; Fiil et al. 1972; Kaplan et al. 1973) and is located on the 50S subunit of the ribosome (Ramagopal and Davis 1974; Richter and Isono 1974; Cochran and Byrne 1974). Ribosomes were used to synthesize the MS nucleotides in vitro (Hazeltine et al. 1972). The MS-synthesizing enzyme is easily dissociated from the ribosome and was found to have a molecular weight of 77,000 daltons (Cochran and Byrne 1974). This enzyme synthesized MSI and II in vitro using GDP or GTP as pyrophosphate acceptors and ATP as the pyrophosphate donor (Cochran and Byrne 1974).

More recently, investigators have mapped another genetic locus which seems to be involved in the metabolic accumulation of MSI and II. It was termed the spotless (Spo) phenotype (Laffler and Gallant 1974). Strains exhibiting this phenotype are characterized by their inability to accumulate detectable levels of MSII, whereas large quantities of MSI do accumulate. This locus, termed Spo T maps

at 72 minutes on the E.coli chromosome. It is a recessive trait and appears to be independent of the RC gene (Laffler and Gallant 1974). Strains carrying this allele show an inhibition of RNA accumulation on amino acid starvation. On the restoration of the missing amino acid, high levels of ppGpp persist in the cells and the normal rate of RNA accumulation does not resume until ppGpp returns to basal levels (Laffler and Gallant 1974; Stamminger and Lazzarini 1974). Recent studies have shown that the Spo T mutation affects both the rate of degradation of ppGpp and the rate of synthesis of its precursor pppGpp (MSII) (De Boer et al. 1977; Leung and Yanazaki 1977).

The finding that ppGpp accumulates when stringent but not relaxed strains of E.coli are starved for an amino acid suggested that ppGpp could be the mediator of the stringent control mechanism, signaling to other metabolic processes in the cell that protein cannot be made. The question therefore arose as to whether ppGpp had any effect on another biochemical process which had been observed to be altered during amino acid deficiency, such as RNA accumulation.

RNA Synthesis

The control of RNA synthesis seems to be the most important feature in the stringent response. It is generally accepted that the accumulation of stable RNA (rRNA and tRNA) is restricted during amino acid starvation (Travers 1974; Gallant and Lazzarini 1976; Dennis 1977). Cashel (1970) suggested that stable RNA synthesis could be regulated by ppGpp *in vivo*. Studies previously discussed, strongly supported an inhibitory role for the MS nucleotides in the regulation of rRNA synthesis. Attempts to show that ppGpp specifically inhibits the synthesis of stable RNA *in vitro* have not been consistent. Reports, based on *in vitro* studies, support the concept that ppGpp inhibits specifically the synthesis of stable RNA (Travers et al. 1975; Travers 1971; Reiness et al. 1975; Van Doyen et al. 1975; Travers 1976; Debenham and Travers 1977). However, in other studies, this effect has not been observed (Hazeltine 1972; Travers 1973; Lazzarini and Johnson 1973; Muto 1975). Travers (1976) has suggested that the problem lies in the type of DNA template used, particularly *E.coli* DNA, such that difficulty arises in

characterizing transcripts other than rRNA which do not respond stringently *in vivo*. In his most recent reports Travers (1976, 1977) showed a preferential inhibition of rRNA and $tRNA^{tyr}$ by ppGpp using Ø 80 DNA carrying a single rRNA or $tRNA^{tyr}$ cistron. Calculated K_i's reported were 150 μM for rRNA and 4 μM for tRNA. Travers has suggested that ppGpp affected the conformation of the RNA polymerase by inhibiting its ability to bind the promoter region.

Recent reports have shown that the synthesis of certain mRNAs for certain proteins is under stringent control, while others are not affected or enhanced. The *in vivo* synthesis of mRNA for ribosomal proteins and elongation factors Tu and G is under stringent control (Dennis and Nomura 1975; Furano and Wittel 1976; Dennis 1977). *In vitro* studies, using DNA from special transducing phages in a coupled transcription-translation system, have shown that ppGpp inhibits the transcription of the genes for ribosomal proteins

and elongation factors Tu and G (Lindahl et al. 1976). Similiar *in vitro* studies have shown that ppGpp also inhibits transcription of the *gal* operon (Parks et al. 1972), and stimulates transcription of the *lac*, *ara*, *trp*, and *his* operons (Parks et al. 1972; Reiness et al. 1975; Stephens et al. 1975).

Thus ppGpp seems to function as both a positive and negative effector of RNA synthesis. This molecule inhibits the transcription of protein synthesizing components, the production of which are not necessary due to the amino acid deficiency. But ppGpp stimulates transcription of messages for proteins involved in amino acid biosynthesis as well those involved in the utilization of other carbon sources (Parks et al. 1972).

Nucleotide Biosynthesis

Studies on the rates of synthesis of nucleotides during the stringent response revealed that soon after amino acid deprivation both the GTP and ATP pools decreased (Gallant et al. 1970; Irr and Gallant 1969). This suggested that nucleotide biosynthesis was inhibited, as one might expect the cessation of RNA synthesis to increase nucleotide pools.

Later studies revealed that two enzymes of the purine biosynthetic pathway are inhibited by ppGpp. These are IMP dehydrogenase which converts IMP to XMP in the first step toward GMP formation (Gallant et al. 1970) and adenylosuccinate synthetase which functions in the IMP to AMP conversion (Gallant et al. 1971). Inhibitory concentrations were well below that which is reached when ppGpp is at maximal levels (Cashel 1970), suggesting that these enzymes are probably inhibited *in vivo*. The control over nucleotide synthesis by ppGpp is possibly to conserve energy and nitrogen (in the form of glutamine) utilized during this process.

Purine and Pyrimindine Transport

Hochstadt-Ozer and Cashel (1972) have shown that purine and pyrimidine transport is inhibited during the stringent response. Using *E.coli* membrane vesicles they showed that ppGpp directly inhibits the uptake of these nucleic acid precursors. Experiments directed towards purine nucleoside transport showed that in *E.coli* the uptake of these precursors

is catalyzed by membrane localized phosphoribosyl transferases. This mechanism involves the cleavage of the ribose from the nucleoside, then the phosphoribosyl transferase transfers ribose 5 phosphate from phosphoribosyl pyrophosphate to the purine base, releasing the nucleoside 5'monophosphate inside the cell. ppGpp was shown to inhibit the purine phosphoribosyl transferases for adenosine and guanosine uptake. The effective levels were within the physiological concentration range. These authors suggest that the inhibition of nucleoside transport during the stringent response could be mediated by ppGpp.

Thus ppGpp not only inhibits RNA accumulation but also the synthesis and transport of precursors for this process. The effect this would have on DNA synthesis is evident; without the necessary precursors DNA synthesis would be inhibited. Sokawa et al. (1970) have shown, that indeed, DNA accumulation is inhibited during the stringent response. This would stop further cell division in auxotrophic strains requiring an amino acid, but only decrease the growth rate until enzymes for amino acid biosynthesis were made in cells having this capability.

Lipid and Phospholipid Metabolism

Sokawa et al. (1968, 1970) showed that the synthesis of lipids is greatly reduced during the stringent response. The question arose as to whether this decrease was directly mediated by ppGpp. A study of this biosynthetic mechanism by Polakis et al. (1973) showed that ppGpp did directly inhibit one of the catalytic components of the acetyl-CoA carboxylase system. Physiological concentrations of ppGpp inhibited the carboxyltransferase reaction. These authors suggested that the stringent control of fatty acid synthesis is mediated through the inhibitory action of ppGpp.

Studies on phospholipid synthesis, also shown to be under stringent control (Golden and Powell 1972), revealed that two enzymes involved in phospholipid biosynthesis were inhibited at physiological ppGpp concentrations (Merly and Pizer 1973). Two enzymes involved are glycerol-3-phosphate acetyltransferase and glycerol-3-phosphate phosphatidyl transferase. The synthesis of lipids and phospholipids for membrane components is an energy-consumming process. Since the cell can no longer make the protein components for membranes, this process

becomes redundant. The control of this process by ppGpp serves to emphasize the diversity of its "domain" and also the interrelationships of the diverse biochemical program taking place in the cell. The necessity for a control mechanism seems only logical.

ppGpp a Complex Regulator

Tomkins suggested that ppGpp falls into the category of a complex regulator (metabolic symbol). It is quite evident that it has a regulatory influence over a variety of seemingly unrelated metabolic processes (domain). Whether it directly affects enzymes in other metabolic processes considered under stringent control such as carbohydrate metabolism or the transport of glycosides remains to be seen. One would expect it does as ppGpp has been shown to stimulate transcription of the *lac* operon which codes for enzymes involved in lactose metabolism. An interesting feature is that this operon as well as a number of inducible enzymes is also regulated by cyclic AMP (discussed later) which is considered a metabolic symbol for carbohydrate utilization.

ppGpp plays the role of a general sensor for amino acid supply, regulating the cell's metabolism with its ability to make protein, the general feature of which is its control over RNA accumulation. One questions whether this unusual nucleotide is a general regulator of RNA synthesis.

Studies have shown that when E.coli is shifted from one carbon source to another, RNA accumulation either increases or stops depending on the amount of energy that can be generated from the carbon source. Going from an energy-rich carbon source to a poorer carbon source results in a cessation of RNA accumulation. Does ppGpp mediate this inhibition of RNA acumulation? Gallant et al. (1976) have shown that a rel^- strain when transferred to a poor carbon source, stopped accumulating RNA immediately after transfer. ppGpp levels, however, remained low and only began to rise 10 minutes after transfer. The maximum ppGpp level reached in this rel^- strain was equivalent to the basal level found in rel^+ strains. Winslow (1971) found ppGpp levels to rise to a high level immediately following down shift in a rel^+ strain, but it subsequently dropped back

to basal levels well before RNA accumulation resumed. These findings suggest that ppGpp may not mediate RNA accumulation. Measurements made on RNA synthesis by Gallant and Lazzarini (1976) indicated that this lack of further RNA accumulation after down-shift may be due to an increased rate of RNA degradation rather than an inhibition of RNA synthesis.

ppGpp in Eukaryotes

Hersko et al. (1971) showed that mammalian cells in culture exhibited similiar responses as bacteria when they were starved of amino acids or deprived of growth promoting substances present in serum. They termed these "pleiotypic responses" and postulated that eukaryotic cells contained a similiar if not identical regulatory compound as ppGpp. The presence of the MS nucleotides in eukaryotic organisms, however, is questionable. Several workers have reported that they had found MS nucleotides in eukaryotic cells (Klein 1974; Horvath et al. 1975;Rhaese 1975; Heizmann 1978). Other laboratories, however, have produced data in conflict of this

conclusion (Jacobson and Lodish 1975; Thammana et al. 1976; Sy et al. 1974; Mamant et al. 1972; Alberghina et al. 1975; Brandhorst and Fromson 1976). This subject has been reviewed recently (Richter and Isono 1977) and the authors concluded that there is no experimental evidence that MSI (ppGpp) is produced in eukaryotic systems.

PHANTOM SPOT

Gallant et al. (1976) reported finding another modified nucleotide in E.coli which they have dubbed the pseudonym phantom spot. The levels of this compound were observed to decrease in response to carbon source or aerobic to anaerobic shiftdown. During such a shiftdown, RNA accumulation ceased. It has been shown such an inhibition of RNA accumulation is not mediated by ppGpp, and is a result of increased rates of degradation of newly-formed RNA (Gallant and Lazzarini 1976).

These authors suggested that the regulation of RNA accumulation might be mediated by the unusual nucleotide phantom spot. They postulated that a portion of the cell's phosphorylating capacity is tapped off to generate the phantom spot nucleotide considered a regulatory facsimile of ATP. The structure of this nucleotide is unknown but is thought to be a guanosine triphosphate with a modified guanine base. The level of this nucleotide is presumed to regulate a number of anabolic processes (domain), such as RNA accumulation, which consume ATP. They

postulated that phantom spot nucleotide affects these processes in a positive manner.

These authors suggested that this modified nucleotide is the symbol of the phosphorylating ability of the cell such that when the rate of phosphorylation falls, a decline of the level of the phantom spot nucleotide brings about a reduction in the expenditure of the cell's ATP. The phantom spot nucleotide, therefore, functions as a buffer by not allowing large fluctuations in the ATP pool or the adenylate energy charge.

CYCLIC AMP IN PROKARYOTES

Occurrence in Prokaryotes

The occurrence of cyclic AMP in bacteria was first reported in 1963(Okabayashi et al. 1963). Since that time it has been observed in a variety of prokaryotes (Rickenberg 1974). Cyclic AMP has been shown to regulate a number of processes in prokaryotes. Cyclic AMP is required for the formation of flagella in coliform bacteria (Yokota and Gots 1970). Cyclic AMP is implicated to be involved in the morphological differentiation process of *Caulobacter cresentus* (Shapiro et al.1972). The most important function of cyclic AMP in bacteria is stimulating the synthesis of proteins under catabolite repression, an example of which is its control over the synthesis of the *lac* operon.

Regulation of Transcription

In bacteria, cyclic AMP has been shown to regulate a number of processes (Robinson et al.1971; Pastan and

Pearlman 1972; Rickenberg 1974) through the stimulation of transcription of messages for proteins, most of which are under catabolite repression.

The best studied systems for which cyclic AMP exerts a regulatory influence is in stimulation of transcription of genes involved in carbohydrate metabolism (Pastan and Pearlman 1972). Tomkins has suggested that cyclic AMP is the metabolic symbol for carbohydrate metabolism. The classic example is the stimulation of transcription of β-galactosidase mRNA by cyclic AMP in the lac operon of E.coli after glucose depletion in a lactose supplemented culture. Transcription of the lac operon is inhibited by the presence of glucose. Cyclic AMP levels remain low but as the glucose is depleted cyclic AMP levels increase. Cyclic AMP binds to the catabolite gene activator protein (CAP). This complex then has an affinity to bind the promoter region of the lac operon. The binding of the cyclic AMP-CAP complex to the lac promotor stimulates the binding of the RNA polymerase enzyme to the gene.

Makman and Sutherland (1965) have shown that if glucose is added back to such a culture cyclic AMP is excreted into the medium. Since E.coli contains the enzyme cyclic AMP phosphodiesterase which degrades

cyclic AMP to AMP, one would expect the cell to degrade the molecule rather than excrete it. Beuttner et al. (1973) have shown, however, that upon glucose readdition, all existing cyclic AMP is excreted, and it continues to be made and excreted into the medium. Thus it would appear that E.coli regulates the cellular level of this molecule by excreting it into the medium. There must therefore be a physiological reason for the excretion process. A plausible hypothesis is that it may influence cell surface functions such as adhesion and solute transport.

THE HPN NUCLEOTIDES REGULATION OF SPORULATION

Highly Phosphorylated Nucleotides (HPN)

Rhaese et al. (1972, 1975) have reported the existence of four unusual nucleotides in the sporulating prokaryote Bacillus subtilis. These compounds have been given the pseudonyms HPNI, II, III, and IV. At least three of these compounds were claimed to exist in various mammalian cell lines grown in culture (Rhaese 1975). Rhaese et al. (1977) reported that these molecules were HPNI, adenosine 3'5' diphosphate (ppApp); HPNII, adenosine 3'diphosphate 5'triphosphate (pppApp); HPNIII, uridine 3'phosphate 5'phosphate-Z-diphosphate (ppZpUp; Z is an undefined sugar); and HPN IV, adenosine 3'5'triphosphate (pppAppp). Rhaese also reported on the presence of MS nucleotides in Bacillus subtilis and showed that they may not directly regulate sporulation as mutants unable to synthesize ppGpp, sporulated normally (Rhaese et al. 1975b).

Rhaese showed that HPNI and HPN II-like compounds

(based on chromatography) were synthesized in vitro by ribosomes from sporulating not vegetative cells. The structures of HPNI and II were elucidated from the ribosomal synthesized products. HPN I and HPN IV-like compounds have been synthesized in vitro using membrane vesicles and ATP as substrate. The products were inferred to be pppAppp (HPN IV) and ppApp (HPN I). It is interesting to note that Oki et al. (1976) have reported the isolation of a purine nucleotide pyrophosphotransferase in the culture medium of five strains of Actinomycetes. This enzyme is capable of synthesizing pppApp or pppGpp.

The structure of HPN III, is only tentative as the authors report they have not been able to isolate large quantities for detailed analysis.

Regulation of Sporulation

Rhaese et al. (1976, 1977) suggested that these unusual nucleotides may be involved with the regulation of sporulation (differentiation) in Bacillus. These workers showed that Bacillus subtilis, when inoculated into a sporulating growth medium, will continue in log phase until the medium cannot support

further growth. At the onset of stationary phase this organism begins differentiating, producing spores about 5 hours later.

Rhaese et al. (1975a) reported that at the onset of sporulation HPN III and IV accumulated rapidly. (Rhaese(1972) initially reported that at this time HPN I, and II accumulated, but more recently (Rhaese et al. 1976) he has disclaimed this and reports an inability to induce the accumulation of HPN I and II in vivo.) Treatments which either delayed (glucose supplementation) or triggered (nitrogen and phosphate starvation) sporulation, similarly delayed or triggered the accumulation of HPN III and HPN IV.

Rhaese and co-workers have also shown that HPN I and HPN II are synthesized by ribosomes from sporulating cells but not by vegetative cell ribosomes. Mutants unable to accumulate HPN IV due to an inability to synthesize this molecule, were unable to sporulate (Rhaese et al. 1977).

Rhaese (1976b) suggested that these HPN molecules accumulated on response to the lack of glucose, phosphate or nitrogen (poor growth conditions) in the medium

(particularly HPN III and IV). He further proposed that nucleotides turned off the genes that were involved with vegetative growth and turned on those that were necessary for differentiation. He presented no enzymological, biochemical or genetic data to support this idea. Thus Bacillus responds to poor growing conditions by the production of spores, this process being regulated by these unsual nucleotides.

It is of interest to note that in 1973 Elmerich and Aubert postulated, based on genetic and biochemical evidence on the mechanism of glutamine inhibition of sporulation in Bacillus subtilis, the existence of an effector which functioned to induce sporulation. The proposed properties of the effector matched some of those of the HPN molecules.

CYCLIC AMP IN EUKARYOTES

Occurrence

Adenosine 3',5'monophosphate (cyclic AMP) was first discovered by Roll et al. (1957) as an intermediate in the hyperglycemic effect of epinephrine and glucagon in animal tissues. It has since been found in a wide variety of eukaryotic organisms such as fungi,plants, and animal tissues (Rickenberg 1974). In most organisms cyclic AMP has been implicated as having a regulatory function. In animal tissues it is commonly referred to as a second messenger due to its intracellular mediation of hormonal regulation (first messenger).

Mediator of Hormone Responses

The mechanism by which cyclic AMP mediates the hormone response was developed by Sutherland as a result of studies on epinephrine and glucagon stimulation of glycogenolysis (Robinson et al. 1971; Sutherland 1972). Hormonal binding to the surface of specific target cells activates adenyl cyclase which catalyzes the synthesis of cyclic AMP from ATP.

This causes a rapid increase in intracellular levels of cyclic AMP, which stimulates the activities of specific protein kinases, which in turn activate or deactivate specific enzymes in glycogen metabolism by phosphorylation. Cyclic AMP levels remain high for only a short time due to the action of cyclic AMP phosphodiesterase which degrades it to 5'AMP. Thus in mammalian tissue cyclic AMP mediates processes by the regulation of protein kinases which in turn function to activate or deactivate proteins by phosphorylation.

Cyclic AMP has been shown to be the intracellular effector for other hormones such as prostaglandins, steroids, vasopressin and thyroid stimulating hormone (Sutherland, 1972). Cyclic AMP is also involved in neurotransmitter function, histone phosphorylation, secretion regulation, muscle contraction and cell morphology (Greengaard and Robinson 1972). It has recently been suggested that cyclic AMP could be a possible regulator of cell proliferation.

Cyclic AMP and Cyclic GMP: Regulation of Cell Proliferation

Hershko et al. (1971) have shown that mammalian cells in culture, when starved of amino acids or deprived of serum, exhibit a simuliar response as the bacterial stringent response. They observed that a variety of

metabolically unrelated biochemical reactions (decrease in RNA accumulation, transport of various RNA precursors, and protein synthesis) respond coordinately in the cell to these environmental changes to regulate the growth "negative pleiotypic response." Conversely, when growth promoting substances (such as insulin)were added to the cell culture, growth was stimulated and an increase in RNA synthesis, protein synthesis and transport was observed. This was termed a positive pleiotypic response. The authors proposed that the negative pleiotypic response was analogous to the stringent response. They postulated that a similiar regulatory mechanism exists in mammalian cells as found in bacteria. However, ppGpp was not found in mammalian cells.

Later studies showed that the negative pleiotypic response could be mimicked by the addition of prostaglandin E_1 to the culture (Kram et al. 1973a) and that these effects could be antagonized by addition of cyclic GMP (Kram and Tomkins 1973b). Since prostaglandin E_1 was known to stimulate cyclic AMP synthesis it suggested that cyclic AMP was the mediator of the pleiotypic response in the regulation of cell growth. Measurements of intracellular cyclic

AMP levels showed that serum deprivation, amino acid starvation and other growth inhibiting treatments of cultured mammalian cells caused a rise in intracellular cyclic AMP levels. Conversely, addition of growth promoting substances giving a positive pleiotypic response caused a corresponding drop in intracellular cyclic AMP levels (Kram et al. 1973b).

Golberg et al. (1974) have shown that fibroblasts in which DNA synthesis has been initiated, have high levels of cyclic GMP and low levels of cyclic AMP. However, in resting cells the reverse was true.

These and other similiar results have led to the proposal that cyclic AMP is a negative effector whereas cyclic GMP is a positive effector of growth, such that cell growth regulation is mediated by these molecules in a "Yin-Yang" fashion (Golberg 1974). However, no data has been reported showing that these two unusual nucleotides have a direct regulatory effect on DNA or RNA synthesis in mammalian cells. One might expect such processes to be regulated by these effector molecules. This subject has been recently reviewed by Pastan et al. (1975) and Goldberg and Haddox (1977).

THE HOT SPOT NUCLEOTIDES

HS 1, 2 and 3 in Fungi

In 1975, LéJohn et al. reported the presence of three unusual polyphosphorylated nucleotides in acid extracts of a variety of fungi. These compounds were given the pseudonyms HS (hot spot) 1, 2 and 3.

The characterization of these compounds has revealed that they are exceedingly complex molecules (McNaughton et al. 1978). HS1 and 2 appear to be uridine dinucleosides with 12 and 10 phosphates respectively. HS3 is a dinucleoside of adenosine and uridine with 8 phosphates. The complete details of the analysis of these compounds is presented in the results section.

These nucleotides are commonly extracted from the cells with acid, however, recent reports suggest that these compounds can be isolated by osmotic shock treatment suggesting that they might also be in close association with the cell membrane (LéJohn et al. 1978).

Possible Regulators of Sporulation

The fungus Achlya has been used in most of the studies related to the physiological importance of the HS compounds. The growth of this organism (see Methods) involves a differentiation stage in which mycelia differentiate at the hyphal tips to spores. HS compounds appear to be possible regulators of this differentiation process.

LéJohn et al. (1978) have shown that just prior to sporulation there is a rapid intracellular synthesis and accumulation of the three HS compounds. The intracellular accumulation of the HS compounds occurred after all the phosphate had been depleted from the medium. Generally HS3 levels are higher than HS2 while HS1 is found in much smaller quantities than either. This accumulation is followed by a rapid decline in cellular HS levels at the time of spore differentiation.

LéJohn et al. (1978) have also observed that Achlya, on being transfered from a complex nutritional medium to a nutrient deficient medium (starvation buffer), induced spored formation. However, addition of glutamine, or various purine and pyrimidine bases to the starvation buffer

repressed or delayed sporulation and HS accumulation. In fact, HS declined. Similarly,in cells transfered to starvation buffer, the intracellular HS levels immediately increased and then declined after a few hours before the cells released the spores. By contrast, in glutamine-supplemented cultures, HS levels did not increase and sporulation was aborted. These authors suggested that the accumulation of the HS compounds was necessary to mediate the sporulation process.

A similiar phenomenon has been observed for the HPN compounds in the sporulation process of Bacillus subtilis (described earlier) suggesting possible similarities in HPN and HS coumpounds. Because of this, it was felt necessary to critically analyze the data pertaining to HPN compounds as reported by Rhaese in recent years (see section on the"HPN compounds: A Critical Analysis").

Regulation of Nucleic Acid Metabolism

Studies measuring the rates of RNA, DNA and HS synthesis over the growth cycle revealed an inverse relationship between the rate of RNA and DNA synthesis

compared to HS synthesis (LéJohn et al. 1975, 1976, 1978) such that as the rate of synthesis of HS compounds increased the rate of RNA and DNA synthesis decreased. This suggested that these compounds might be negative effectors of both DNA and RNA synthesis.

In vitro experiments showed that the HS compounds were potent inhibitors of DNA dependent RNA polymerases isolated from Achlya (McNaughton et al. 1975; LéJohn et al. 1978). HS3 and 2 were found to be equally potent effectors, completely inhibiting the various enzymes at concentrations within the physiological range. These compounds were also shown to regulate the in vitro activity of ribonucleotide reductase isolated from Achlya (Lewis et al. 1977). This enzyme catalyzes the synthesis of deoxnucleotides from ribonucleotides for DNA synthesis. HS3 was found to be more potent than HS2. Inhibition was observed in the physiological concentration range.

The effects of the three HS compounds on a partially purified preparation of DNA polymerase(s) were tested and it was reported that only HS1 (not HS2 or 3) powerfully inhibited this (these) enzyme(s). Complete inhibition was observed at concentrations of

5 µg/ml (LéJohn et al. 1978) suggesting that HS1 could function as a mediator of DNA synthesis at the polymerase level.

Sensors of Nutrient Availability

LéJohn et al. (1978) have suggested the possibility that HS compounds might be sensors of the availability of specific nutrients such as nitrogen or phosphate in the growth environment. Recent reports have shown that the HS compounds can be removed from the cell by cold osmotic shock treatment suggesting that these compounds are in close association with the cell membrane. In view of this location, the question has been raised as to the possible involvement of HS compounds in regulation of transport (Stevenson and LéJohn 1978).

In summary, the data suggest that these compounds play an important role in the regulation of growth and development in Achlya possibly through the control of nucleic acid metabolism. Similar studies with HS3 in mammalian cells would seem to support this concept of a role for HS3 in the regulation of nucleic acid metabolism.

HS3 in Mammalian Cells

Goh and LéJohn (1977) have reported the presence of HS3 in various mammalian cells in culture. Studies have revealed that both fungal and mammalian HS3 molecules are identical (Lewis et al. 1977).

Physiological studies have revealed that mammalian HS3 accumulates when glutamine was removed from the growth medium (Goh and LéJohn 1977, Goh et al. 1977). Other amino acids, when removed from the medium did not cause HS3 accumulation. Also a decrease in the rate of DNA and RNA synthesis was observed during glutamine starvation of chinese hamster ovary cells suggesting that HS3 might regulate these processes here as in fungal cells.

Experiments with various drugs (inhibitors of purine and pyrimidine biosynthesis) and mutants with defective de novo and salvage pathways of nucleotide biosynthesis revealed that mammalian HS3 accumulated as a result of either the lack of, or an imbalance in the synthesis of precursors for RNA and DNA production. It was concluded that the accumulation of HS3 during glutamine starvation may be due to the inhibition of purine and pyrimidine biosynthesis which are glutamine-requiring processes. Thus HS3 appears to accumulate in response to deficiencies

of nutrients for nucleic acid biosynthesis.

Studies with both Achlya and mammalian cell HS3 have shown them to be equally effective inhibitors of DNA-dependent RNA polymerases (S.H. Goh Ph.D. Thesis) and ribonucleotide reductases (Lewis et al. 1977) isolated from CHO cells. Inhibitory concentrations were within the physiological range. Since RNA and DNA synthesis are inhibited in vivo during glutamine starvation, the authors suggest that these processes might be mediated by HS3 in vivo.

DIADENOSINE TETRAPHOSPHATE

Rapaport and Zamecnik (1976) have reported the presence of the unusual nucleotide diadenosine 5',5'''-p^1,p^4 tetraphosphate (AppppA) in various mammalian cell lines grown in culture and also mouse liver tissue. This molecule can be synthesized in the back reaction of the amino acid activation step (Zamecnik et al. 1966).

These authors have shown that the cellular levels of the molecule decrease 10-fold upon amino acid starvation, serum deprivation, drug inhibition of protein synthesis or inhibition of DNA synthesis with hydroxyurea. Cellular concentrations ranged from 0.05-5μM. All the above treatments were considered too slow to effectively stop further growth of the cells in culture.

Grummt (1978) has recently reported a stimulation of initiation of DNA synthesis in resting baby hamster kidney cells by AppppA when incubated in an in vitro DNA-synthesizing system. However, the concentration of AppppA needed to stimulate replication in vitro was about 50-fold greater than the physiological levels reported by Rapaport and Zamecnik.

These authors suggest that this molecule may be a positive effector of cellular proliferation; high levels which symbolize optimum growing conditions, stimulate DNA synthesis. However, when necessary components for growth are removed from the medium (serum factors or amino acids) the levels of this nucleotide drop in conjunction with a cessation of growth.

A similiar function has been proposed for cyclic GMP in mammalian cells (discussed previously) however, no direct evidence has been reported showing that cyclic GMP regulates DNA synthesis.

HPN COMPOUNDS: A CRITICAL ANALYSIS

Due to possible similarities of the Highly Phosphorylated Nucleotides (considered mediators of sporulation in Bacillus subtilis) and the HS compounds (considered possible regulators of sporulation in the eukaryotic fungus Achlya) it was considered necessary to critically review the literature pertaining to the HPN compounds as there are conflicts in the studies reported to date which require resolution.

In 1972 Rhaese et al. reported the presence of two unusual nucleotides in acid extracts of Bacillus. These were designated HPN I and II. These two nucleotides were reported to accumulate concomitantly with the onset of sporulation. No characterization was described, however it was reported that acid hydrolysates of HPN I and II, partially purified from the acid extractable material, released ultraviolet absorbing entities which comigrated with uridine or thymidine when chromatographed on paper. Then in 1975, Rhaese et al. (1975a) reported that HPN I and II were synthesized by ribosomes from sporulating cells. The products of ribsome -mediated

synthesis were deduced as ppApp (HPN I) and pppApp (HPN II).

In the same report (Rhaese et al. 1975a) the authors reported the existence of two other unusual nucleotides HPN III and IV. These two nucleotides (HPN III and IV) were reported to accumulate at the onset of sporulation. The tentative structure of HPN III was given as ppUpp and HPN IV was pppAppp.

Therefore, according to Rhaese we have four unusual compounds HPN I, II, III, and IV all of which accumulate prior to sporulation. However, to complicate matters, Rhaese et al. (1976b) reported their inability to show the presence of HPN I and II in vivo (ie. ppApp and pppApp). This indicated that only HPN III and IV accumulated at the onset of sporulation. Therefore, it appears that only two nucleotides accumulate at this stage of growth in vivo. These are now designated HPN III and HPN IV.

The characterization of the acid extractable material has not been reported except for the result showing that two nucleotides which accumulated prior to sporulation both contained an ultraviolet absorbing entity which comigrated with uridine or thymidine.

The tentative structure of HPN III has been

given as ppZpUp where Z is an undefined sugar. Rhaese et al. (1977) reported that they had not been able to isolate sufficient amounts of this compound to chemically analyze it but they considered it to be uridine polyphosphate.

The structure of HPN IV has been reported as pppAppp. This structure was obtained from the product obtained from a membrane enzyme catalyzed reaction utilizing ATP as substrate (Rhaese et al. 1977). This compound contains adenosine, however the product isolated from acid extracts was reported to contain a nitrogen base which comigrated with uridine or thymidine.

It is evident that these inconsistencies have arisen because of problems in isolating and purifying the acid extractable compounds in large enough quantities. No conclusions can be drawn, however, with respect to the similarity between the HS nucleotides and the HPN compounds, based on available data. It is evident that similarities do appear with respect to data pertaining to their involvement in the sporulation process of the eukaryote Achlya and the prokaryote Bacillus. To conclude that they are similiar compounds is premature, and will have to

await a more complete definition of the HPN compounds as well as HS nucleotides.

MATERIALS AND METHODS

MATERIALS AND METHODS

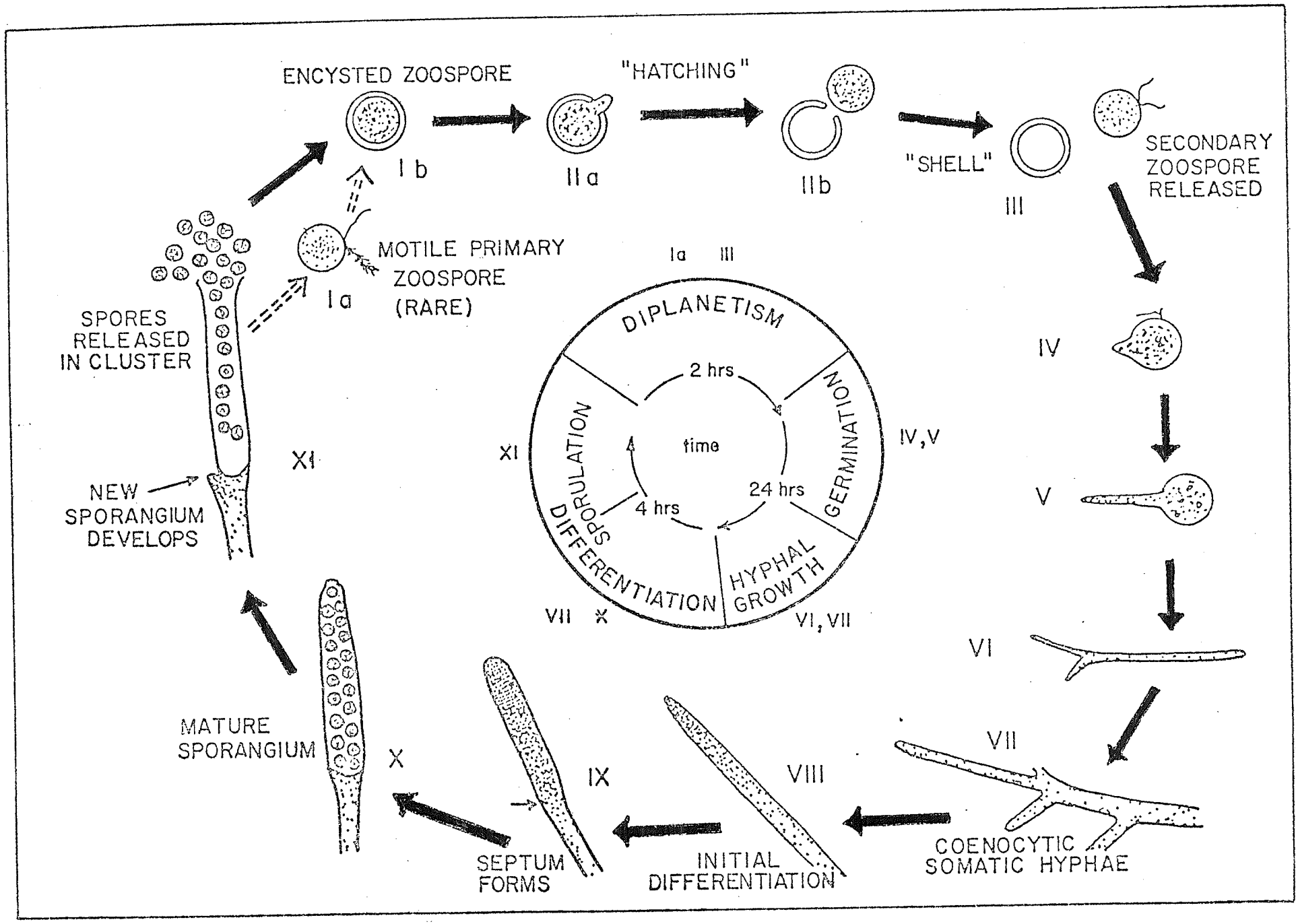
A. ORGANISMS AND CULTURE CONDITIONS

1. Achlya

The fungal strain employed throughout this study was obtained from Dr. J.S. Lovett, Purdue University, and has been described and designated Achlya sp. (1969) by LéJohn and Stevenson (1970).

Achlya sp. 1969 is described as follows: class, oomycetes; order, saprolegniales; and family, saprolegniaceae (Alexopoulous, 1962; Dick, 1973). Fungi of this family have a eucarpic thallus construction which is filamentous, mycelial and non septate. They are found in fresh water environments feeding on plant and animal debris. Members of the family saprolegniaceae exhibit sexual and asexual reproduction. The sexual cycle has been extensively studied with regard to hormonal regulation of the process (Barksdale 1969). Achlya sp. 1969 exhibits only an asexual reproductive mode during which the hyphal tips differentiate producing sporangia and zoospores. The developmental stages of the asexual life cycle have been described by Cameron and LéJohn (1972)

Figure 1: A schematic summary of the asexual life cycle of Achlya sp. 1969. Reproduced from R.M. Stevenson Ph D. Thesis.


ENCYSTED ZOOSPORE
"HATCHING"
"SHELL"
SECONDARY ZOOSPORE RELEASED
Ib
IIa
IIb
III
IV
V
VI
VII
VIII
IX
X
XI
Ia
MOTILE PRIMARY ZOOSPORE (RARE)
SPORES RELEASED IN CLUSTER
NEW SPORANGIUM DEVELOPS
MATURE SPORANGIUM
SEPTUM FORMS
INITIAL DIFFERENTIATION
COENOCYTIC SOMATIC HYPHAE
DIPLANETISM
2 hrs
GERMINATION
time
24 hrs
HYPHAL GROWTH
4 hrs
SPORULATION DIFFERENTIATION
Ia III
IV,V
VI, VII
VII *
XI

as illustrated in Figure 1 with Roman numeral designations. Achlya sp. 1969 is diplanetic in nature, having both a primary (stage 1b) and secondary zoospore (III) as is a characteristic of the saprolegniaceae. The secondary zoospore is freely motile and begins germinating by growing a thin germ tube (IV) which elongates to form a hyphae (V). The hyphal tube elongates and branches (VI and VII) but remains coenocytic. Sporangial development (differentiation) becomes visible by the darkening of the hyphal tips which is the result of multi-nuclei and cytoplasmic aggregation at this location (VIII and IX). A septum then forms delimiting the sporangium from the rest of the hypha and cytokinesis results with each nucleus becoming enwrapped with a portion of the cytoplasmic material to form cysts (X). Eventually, the sporangium matures and lyses at the tip releasing a cluster of 20-30 spores (cysts) and a new sporangium begins development at the base of the emptied sac (XI).

2. Growth Media and Conditions for Achlya

Growth Medium: Achlya sp. was routinely grown on G_2Y medium which consisted of 5.0g glucose and 0.5g yeast extract per litre of tap distilled water.

Plate Cultures: Stock cultures were grown in 20ml volumes of liquid G_2Y medium in 9cm diameter plastic Petri dishes at room temperature. Inoculum for the plates was 2ml of a spore suspension prepared by shaking week-old hyphal mats from G_2Y plates in flasks of sterile distilled water (approximately three mats per 100ml).

Roux Culture: To obtain a large spore inoculum for carboy cultures, Roux bottles containing 80ml of G_2Y medium were innoculated with 10ml of a spore suspension prepared as described for plate cultures. These were incubated for 48 hours at room temperature.

Carboys: To obtain large volumes of hyphal material for nucleotide extraction procedures, cultures were grown in carboys under forced aeration. Carboys contained 15 litres of G_2Y medium and were inoculated asepticaly with a spore suspension prepared by pouring the contents of six Roux bottle cultures (after vigourous shaking of each Roux bottle) through

four layers of cheese cloth into a 2.8 litre Fernback flask, The average numer of spores inoculated in 15 L medium was 3×10^9. These cultures were routinely incubated at 28° in large water baths for approximatley 12 hours then harvested by suction filtration on Whatman #1 filters. The cells were washed with tap distilled water and treated with 1N formic acid to extract HS compounds.

Small Scale Cultures: To obtain small quantities of cells for HS extraction by the osmotic shock procedure (see below), cells were grown in stoppered erlenmeyer flasks containing 500ml G_2Y medium. The spore inoculum was prepared by pouring mycelial mats and spent medium from 10 petri plate cultures (week-old) into a sterilized stoppered erlenmeyer flask, shaking vigorously, and then filtering the suspension through 61µm (pore size) nylon mesh cloth. These cells were grown for 21 hours at 21°, harvested by suction filtration (not to dryness) and suspended in starvation buffer.

3. Chinese Hamster Ovary Cells

Chinese hamster ovary (CHO) cells derived from the original CHO-K1 of Puck et al. (1958) were generously provided for this study by Dr. J.A. Wright of the University of Manitoba. They were routinely cultured as described by Goh and LéJohn (1977). Chinese hamster ovary cells used for the extraction of HS3 were kindly provided by S.H. Goh.

B. METHODS

1. ^{32}P-labelling and Extraction of HS compounds from *Achlya*

To obtain HS compounds labelled with ^{32}P-radioactivity a small 3 litre carboy containing 2.5 litres of G_2Y medium was supplemented with ^{32}P-orthophosphate (2-4 mCi). The carboy was inoculated with a filtered spore suspension (5 x 10^8 spores) and grown for 12 hours at 28°. The cells were collected and extracted with ice cold formic acid (1M) for 1 h at 4°C. Normally 1ml of acid was used per gram wet weight of *Achlya*. This mixture was then filtered to obtain the ^{32}P-labelled formic acid extractable nucleotides.

2. Partially Purified HS Compounds

The method for partial purification of HS compounds has been described (LéJohn et al. 1975). ^{32}P-labelled extract was chromatographed on PEI-cellulose thin layer plates in solvent I. The plates were exposed for 2-4 hours to X-ray film. The areas corresponding

to ^{32}P-labelled HS compounds were cut out and eluted from the PEI-cellulose with 2M triethylammonium bicarbonate (TEAB) buffer pH 8.0, after the plates had been rinsed for 15 min in methanol to remove phosphate. The liquid was filtered to remove cellulose particles, evaporated to dryness under vacuum, and washed with water several times before resuspending the ^{32}P-labelled HS compounds in deionized water.

3. Starvation Procedure and Labelling with ^{14}C-mannitol

Small (500ml) cultures of Achlya were harvested, washed with water and resuspended in 20 ml of starvation buffer (1mM tris-HCl pH 8 and 0.1mM $CaCl_2$) supplemented with 50 µCi of ^{14}C-mannitol, and incubated for 5 h at 21°. Under these conditions differentiation occurs approximately 4-5 h after inoculation (LéJohn et al. 1978). The cells were collected by filtration on Whatman #1 filter discs, and the cells were osmotically shocked to obtain ^{14}C-HS compounds. The osmotic shock procedure used has been described (Cameron and LéJohn 1972; LéJohn and Cameron 1973) and consists

of suspending the cells in 10ml of a hypertonic solution of 0.5M sucrose, 0.05M tris-acetate, pH 8 and 0.1M EDTA. The cells were incubated at 30° for 15 minutes, then collected by filtration on SMWP-Millipore filters and resuspended in 2ml of ice cold 0.5mM $MgCl_2$. This mixture was agitated vigorously and kept on ice for 10 minutes. The cells were centrifuged at 10,000 x g for 10 minutes at 0°, the supernatant (osmotic shock fluid) collected and treated as described in the pertinent sections.

4. Chromatography

Paper chromatography was performed with Whatman No. 1 and orange ribbon C (Schleicher and Schuell Co.) paper. Thin layer chromatography was performed with polyethyleneimine (PEI) cellulose and thin layer cellulose (Cel 300/uv) plastic-backed sheets obtained from Brinkmann. The solvents used were:

solvent I: 1.5M KH_2PO_4, pH 3.6 (modified solvent I consisted of 1.0M KH_2PO_4, pH 3.6).

solvent II: 3.3M ammonium formate in 4.2% boric acid adjusted to pH 7 with concentrated NH_4OH.

solvent III: 200ml each of 0.5M Na_2HPO_4 and NaH_2PO_4, 20ml n-propanol, 1200g ammonium sulphate brought to 2 lires with distilled H_2O.

solvent IV: l-butanol: isobutyric acid: H_2O: ammonium hydroxyde (10:5.3:3:0.3).

solvent V: saturated ammonium sulphate:0.1M sodium acetate: isopropanol (75:19:2).

solvent VI: isopropanol: formic acid: H_2O (80:4:20).

solvent VII: n-propanol:H_2O (7:3).

solvent VIII: ethyl-acetate: acetic acid: formic acid: H_2O (9:1.5:0.5:2).

solvent IX: n-butanol:acetic acid:H_2O (3:1:1).

solvent X: ethyl-acetate: pyridine: H_2O (top phase) (2:1:2).

5. Nucleotide, Nucleoside and Nucleobase Analysis

HS compounds (1 A_{260nm} unit) were digested by chemical and enzymatic methods (described in text) and analyzed for uv-light absorbing components by chromatography. One dimensional paper chromatography was performed with orange ribbon C paper in solvent III,

or on PEI-cellulose thin layer plates in solvent I and modified solvent I. One and two dimensional chromatography was carried out on thin layer cellulose sheets with solvents IV and V. Localization of products was by uv-light absorption. Phosphorylated substances were detected and identified after chromatography by autoradiography when ^{32}P-labelled HS compounds were used. Kodak RP-14 Royal X-Omat films were exposed to chromatograms for 24-48 h (in the case of ^{14}C-labelled compounds longer exposure times were used) before development. The ^{32}P contents of HS compounds and various products were quantitated by cutting the corresponding areas on the chromatogram and determining the radioactive content by use of liquid scintillation.

6. Spectral Analysis

The HS compounds and fragments produced by chemical and enzymatic digestion, were spectrally analyzed at pH 1 using 0.01N HCl, pH 7 in 0.01M sodium phosphate buffer and at pH 12 in 0.01N KOH. Absorbances at 260nm were also obtained for HS compounds in deionized water.

7. Liquid Scintillation

Radioactive samples were estimated by (both ^{32}P and ^{14}C labelled substances) liquid scintillation counting techniques. Cuttings from chromatograms, or liquid samples were placed in glass scintillation vials, and 5ml of liquid scintillation mixture prepared according to Bray (1960) was added. The composition of the scintillation fluid was: naphthalene (60g/l), PPO (4g/l), POPOP (0.2g/l), ethylene glycol (20ml/l), methanol (100ml/l), and p-dioxane to 1 litre. Radioactivity was determined with the use of a Beckman Model LS 230 liquid scintillation spectrometer, with external standard corrections.

8. Phosphate Analysis

Total organic phosphate was estimated after ashing by the method of Lowry et al. (1954). Periodically non-ashed samples were used for controls. Authentic AMP and KH_2PO_4 were used as standards. Normally duplicate samples were analyzed, the results were averaged, and expressed as nmoles phosphate.

^{32}P-labelled HS components produced by degradation of HS compounds were located by autoradiography and the ^{32}P content estimated by liquid scintillation as described earlier.

^{32}P radioactivity of HS or components fractionated by column chromatography were measured by the cerenkov method (Parker and Elrick 1970). Fraction volumes of 0.4 to 1.0ml were placed in small plastic-capped tubes inserted into glass scintillation vials and the samples counted by a Beckman model LS 230 liquid scintillation spectrometer.

9. Amino Acid Analysis

Approximately 1 A_{260} nm unit of HS compounds were hydrolyzed in a sealed tube with 6N HCl at 105^{o} for 20 h (or conditions specified in text) after which excess acid was removed by evaporation in vacuo. The residue was washed several times with water and redissolved in a small volume of deionized water. Analysis was performed by two dimensional thin layer chromatography on cellulose plates with solvent VI in the first dimension and solvent VII

in the second dimension. Localization of amino acids on paper was accomplished by using freshly prepared ninhydrin spray (3% in ethanol) with overnight incubation to allow spots to appear. Quantitation and identification of amino acid content was also carried out with a Beckman model 121 amino acid analyzer (courtesy of the Plant Science Department, University of Manitoba).

The optical isomeric form of the amino acid was determined using D- and L- amino acid oxidases (Sigma Chemical Co.). About 3 A_{260} nm units of HS3 was hydrolyzed and approximately 1 A_{260} unit of hydrolysate was treated with the specific oxidase (1µg/ml) in citrate buffer. Samples were then chromatographed one dimensionally in solvent VII and amino acids located by ninhydrin spray.

10. Carbohydrate Analysis

Ribose was quantitated by the orcinol procedure (Brown 1946). For the estimation of ribose in uridine the procedure of Haavaldesen et al. (1959) was followed with minor changes described in the pert-

inent sections on "Carbohydrate Analysis". Sugars were also estimated by the periodate consumption method described by Dixon and Lipkin (1954) and the periodate oxidation-formaldehyde determination procedure described by Burton (1957). The amino sugar content was estimated by the ninhydrin based reaction described by Moore and Stein (1954), using glucosamine as standard. In all tests duplicate samples were analyzed and averaged.

Prior to sugar analysis by chromatography, 5-10 A_{260} nm units or equivalent of HS3 (or fragments) were hydrolyzed *in vacuo* with 1N HCl at 105°C for 20 h. Excess acid was removed by evaporation, the residue redissolved in water and mixed with activated Dowex-50. After centrifugation for 10 min, the supernatant was removed, evaporated to dryness and dissolved in 25µl of distilled H_2O. Samples were chromatographed one-dimensionally on Whatman No. 1 paper in either of solvents VII, IX or X. Localization of sugars on paper was performed with specific indicators as described (Bailey 1969).

The indicators were:

M1: sodium periodate-permanganate; 2% aqueous Na-metaperiodate: 1% $KMnO_4$ in 2% aqueous Na_2CO_3 (4:1).

M2: p-anisidine; 3% p-anisidine hydrochloride in BuOH-EtOH-H_2O (4:1:1) plus a trace of $SnCl_2$.

M3: bromocresol purple; 0.04% bromocresol purple in 95% methanol containing 0.1% boric acid + 7.5ml 1% borax solution per 100ml. Sugar alcohols give yellow spots on blue ground.

M4: vanillin; 1% vanillin in ethanol: 3% aqueous perchloric acid (1;1). Polyhydric sugar alcohols give pale grey-blue spots.

M5: benzidene-TCA; 0.5g benzidene in 20ml acetic acid plus 80ml ethanol.

M6: ninhydrin; 0.05% ninhydrin in BuOH.

11. Column Chromatography

Columns of superfine Sephadex G10, G50 and A25 were used to fractionate chemical and enzymatic digests of HS3 as well as intact HS compounds.

Sephadex G10 columns were used to desalt the separated components (fractions pooled and evaporated to 1-2 ml) after Sephadex A25 fractionation. The Sephadex G10 columns were pre-equlibrated with 50 mM TEAB buffer pH 8. Fractions containing desalted components were pooled, evaporated to dryness, washed several times with water and resuspended in known volumes of distilled water. These were stored at -20^{o}.

Sephadex G50 columns (1x100cm) pre-equilibrated with 50 mM TEAB buffer pH 8 were used for relative sizing of the HS compounds.

12. Enzymes

HS compounds or their component parts were hydrolyzed by a variety of enzymes which were purchased from Sigma Chemical Co. The details of the various enzyme procedures are outlined in pertinent legends and in the text. The enzymes used were:

1. Nucleotide pyrophosphatase;(E.C. No. 3.6.1.9.) from Crotalus atrox venom, specific activity 4.9units/mg protein.
2. Bacterial alkaline phosphatase; (E.C. No. 3.1.3.1.) from E.coli , specific activity 30 units/ mg protein.
3. Bovine spleen phosphodiesterase; (E.C. No. 3.1.4.18) specific activity 10 units/ mg protein.
4. Snake venom phosphodiesterase; (E.C. No. 3.1.4.1.) from Crotalus adamanteus venom, specific activity 0.2 units/ mg protein.
5. D-amino acid oxidase;(E.C. No.1.4.3.3)from hog kidney, specific activity 17 units/ mg protein.
6. L- amino acid oxidase; (E.C. No. 1.4.3.2)from Crotalus adamanteus venom(typelV), specific activity 6.2 units/ mg protein.

13. Source of Materials

Common biochemicals were obtained from Sigma Chemical Co. ^{32}P-orthophosphate (S.A. 88-122 Ci/mg) and ^{14}C-mannitol (S.A. 60 mCi/mmol) were obtained

from Amersham-Searle. Sephadex G10, G50 and A25 were obtained from both Sigma and Pharmacia Chemical Co. PEI-cellulose and cellulose thin layer plates were purchased from Brinkmann and vials for liquid scintillation counting were obtained from Fisher Scientific Co. Membrane filters were purchased from Millipore Corp. All other bench chemicals were obtained from Fisher Scientific.

RESULTS

RESULTS

A. THREE UNUSUAL NUCLEOTIDES: HS1, 2 and 3

The existence of three novel nucleotides in acid extracts of Achlya and other fungi was reported by LéJohn et al., (1975). These compounds were given the pseudonyms HS1, HS2 and HS3 on the basis of their migratory properties when chromatographed in one dimension on PEI-cellulose thin layer plates in 1.5M phosphate buffer pH 3.65 (Fig. 2a). This system is similar to that developed by Cashel (1969) for the separation of the MS nucleotides except that Cashel used a buffer pH of 3.4. Two dimensional chromatography of the formic acid extract is shown in figure 2b. This system (developed by Cashel) shows the three HS compounds migrating slower than GTP in both dimensions. The MS nucleotides (kindly provided by Dr. M. Cashel) when cochromatographed with labelled acid extract from Achlya did not comigrate with any of the HS compounds two dimensionally (for R_f values see table 1) indicating the HS compounds are not identical to the MS nucleotides.

Figure 2: One and two dimensional chromatography of ^{32}P-radioactive labelled formic acid extract from 12 h Achlya mycelia. (a) is an autoradiogram of a one dimensional chromatograph on PEI-cellulose in solvent I; (b) is a two dimensional separation of the same extract chromatographed in solvent II (first dimension) and solvent I (second dimension). After chromatography in the first dimension the plate was washed in methanol for 15 min, then developed in the second dimension.

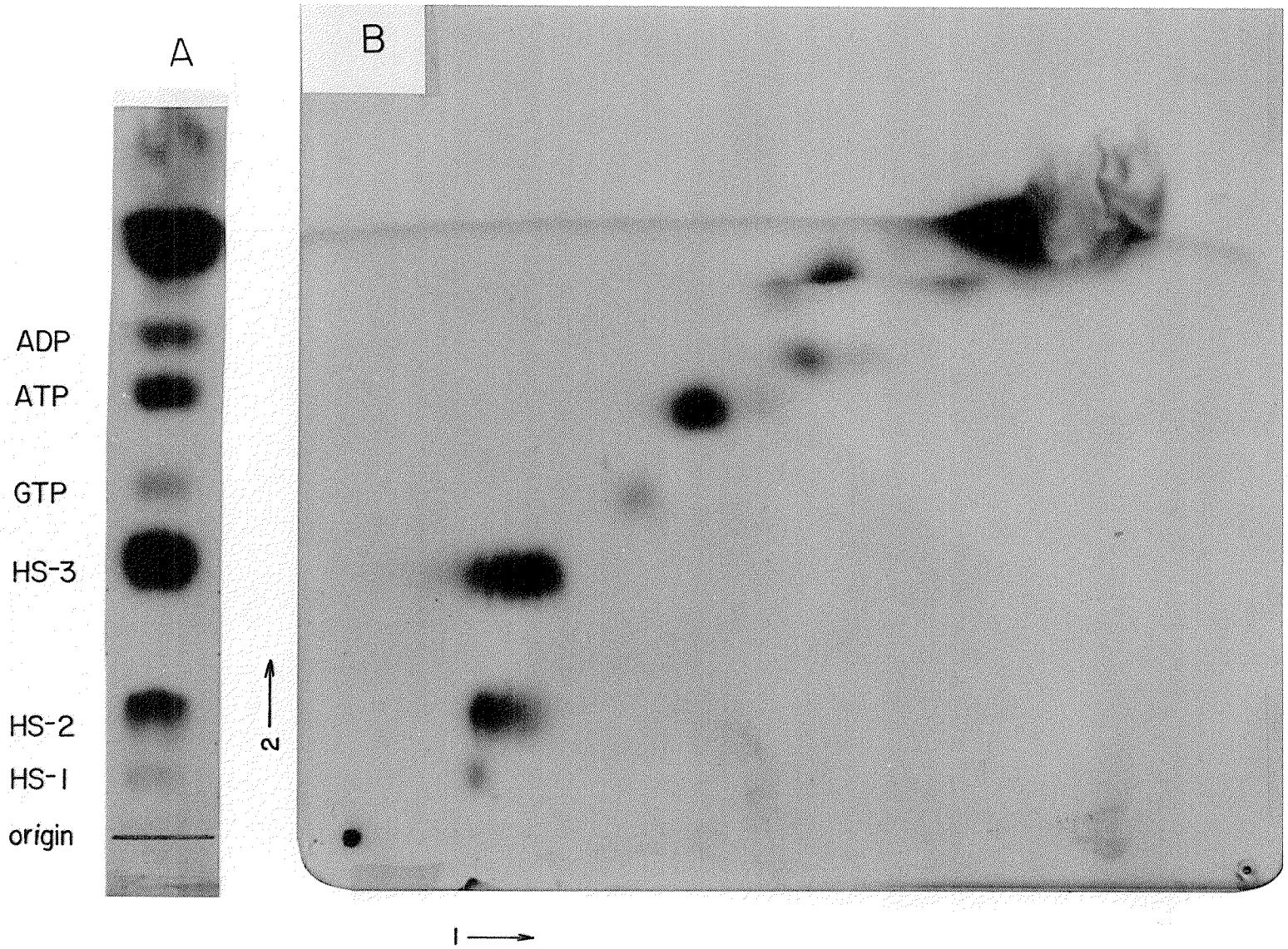

A
B
ADP
ATP
GTP
HS-3
HS-2
HS-1
origin
2
1

Table 1: R_f values of nucleotides

Nucleotide	Rf value dimension 1	2
HS3 (Achlya)	0.22	0.35
HS2	0.17	0.17
HS1	0.17	0.08
HS3 (mammalian)	0.21	0.36
ppGpp (MS1)*	0.33	0.28
pppGpp (MS2)*	0.29	0.17
ATP*	0.46	0.58
GTP*	0.38	0.45

Separation in the first dimension used solvent II and second dimension used solvent I.

* located by uv-light absorption

A similar compound as HS3 was found in acid extracts of mammalian cells grown in culture (Goh and LéJohn 1977). Figure 3(a) shows an autoradiogram of a one dimensional separation of an acid extract from mammalian cells labelled with ^{32}P-orthophosphate. Mammalian cell HS3 comigrates with Achlya HS3 in one dimension. The level of mammalian cell HS3 increased when the cells were starved of glutamine as is shown in figure 3(b) (courtesy of S.H. Goh). Figure 4 is an autoradiogram of a two dimensional separation of ^{32}P-labelled acid extract from Chinese hamster ovary cells starved of glutamine for 2 h. The mammalian HS3 comigrates in both dimensions(for Rf values see table 1) with HS3 from Achlya.

Studies with HS compounds from Achlya and HS3 from mammalian cells have shown them to regulate the in vitro activities of DNA-dependent RNA polymerases(McNaughton et al. 1975; S.H.Goh, Ph.D. Thesis) ribonucleotide reductase (Lewis et al. 1976, 1977) and a mixture of DNA-dependent DNA polymerases (LéJohn et al. 1978).

The importance of these compounds as possible mediators of nucleic acid metabolism in eukaryotes, instigated this investigation which deals with the

Figure 3: Autoradiograms of 1M formic acid extracts of wild type Chinese hamster ovary cells grown in ∝minimal medium supplemented with ^{32}P-orthophosphate for 2 h (A) with L-glutamine and (B) without L-glutamine.
Acid extracts were chromatographed on PEI-cellulose plates in 1.5M phosphate buffer (ascending chromatography).

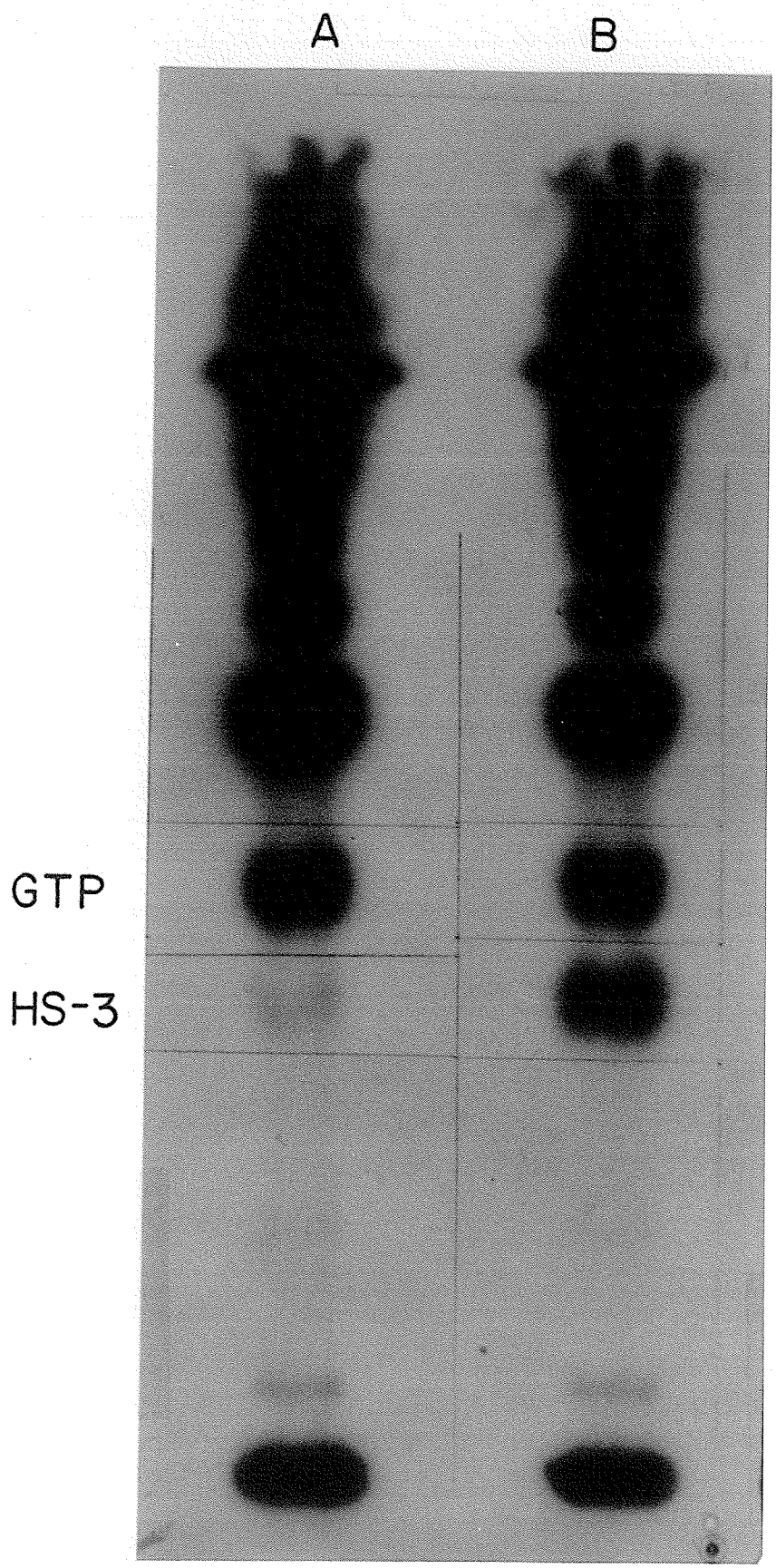
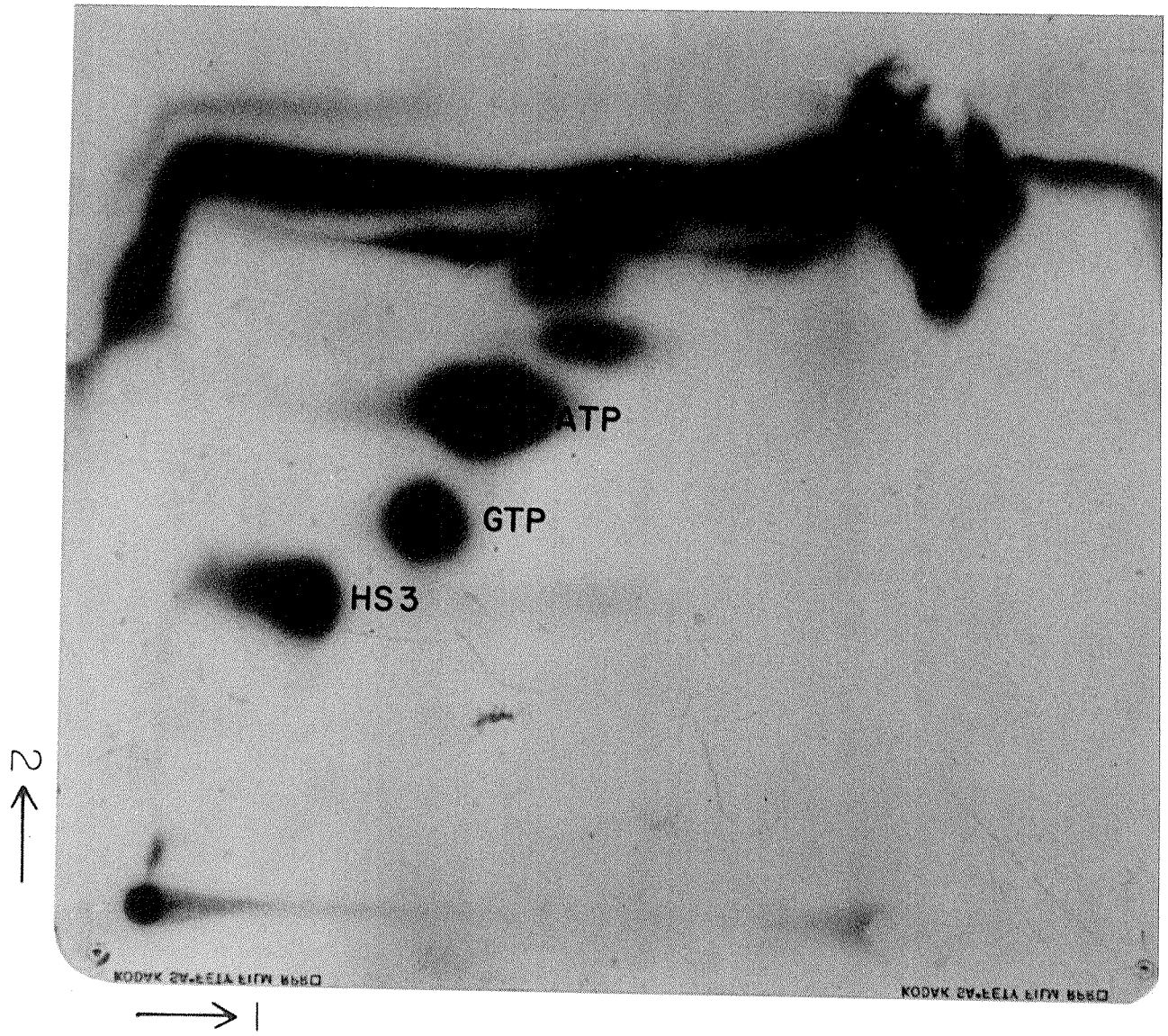

A
B
GTP
HS-3

Figure 4: Two dimensional autoradiogram of 1M formic acid extract of wild type Chinese hamster ovary cells labelled with ^{32}P-orthophosphate and starved of L-glutamine for 2 h. The first dimension was developed in solvent II, the second dimension was developed in solvent I.


ATP
GTP
HS3
2
1

purification and characterization (partial) of these unusual nucleotides.

B. ISOLATION AND PURIFICATION OF HS COMPOUNDS

1. From Achlya

The procedure for the purification of HS compounds has been described (McNaughton et al. 1978). For large scale preparation of HS compounds, Achlya was grown in 15 litre cultures under forced aeration at 28^{o} for 12 h before harvesting and extracting HS compounds from the mycelia (see Materials and Methods). Pulse labelling studies showed that under these conditions there was a rapid increase in the rate of $^{32}P_i$ incorporation into HS compounds that reached a maximum at approximately 11 h from the time of inoculation (Fig. 5). A study of the relative pool sizes of acid extractable HS1, 2 and 3 (represented as HS/GTP ratio in figure 6) showed that the levels of these compounds reached a maximum at approximately 11½ to 12 h before declining to basal levels. These results suggested that for optimal recovery of acid extractable HS compounds, the cells had to be harvested at 12 h. If the cells were harvested much later very little acid extractable HS was obtained (figure 5).

Figure 5: Rate of entry of ^{32}P-orthophosphate into HS3 (O), HS2 (Δ) and HS1 (□) during the growth cycle of Achlya. Twenty-five ml cell suspensions from carboy culture (see Methods) were pulse labelled for 10 min with ^{32}P-orthophosphate. The sample was filtered and HS extracted with 1M formic acid. ^{32}P-HS compounds were determined as described in Methods. Dry weights (●) were determined from an equivalent cell suspenison after filtering and drying at 80° for 20 h.

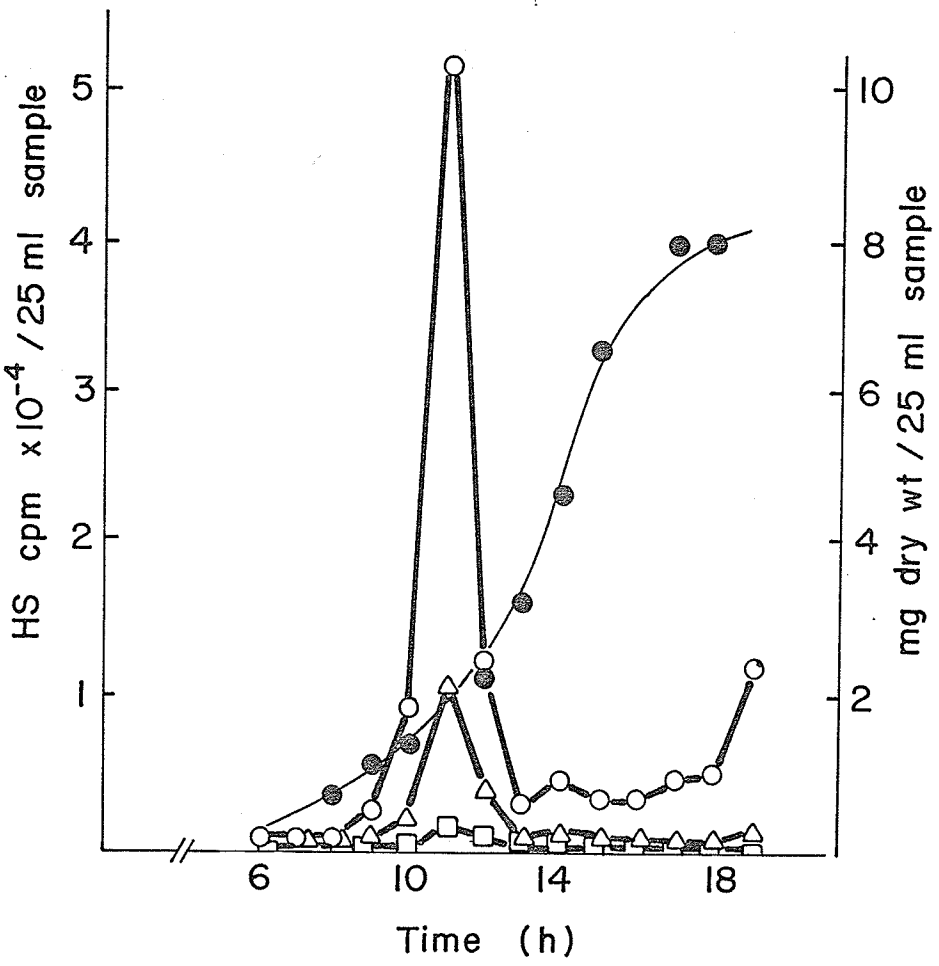
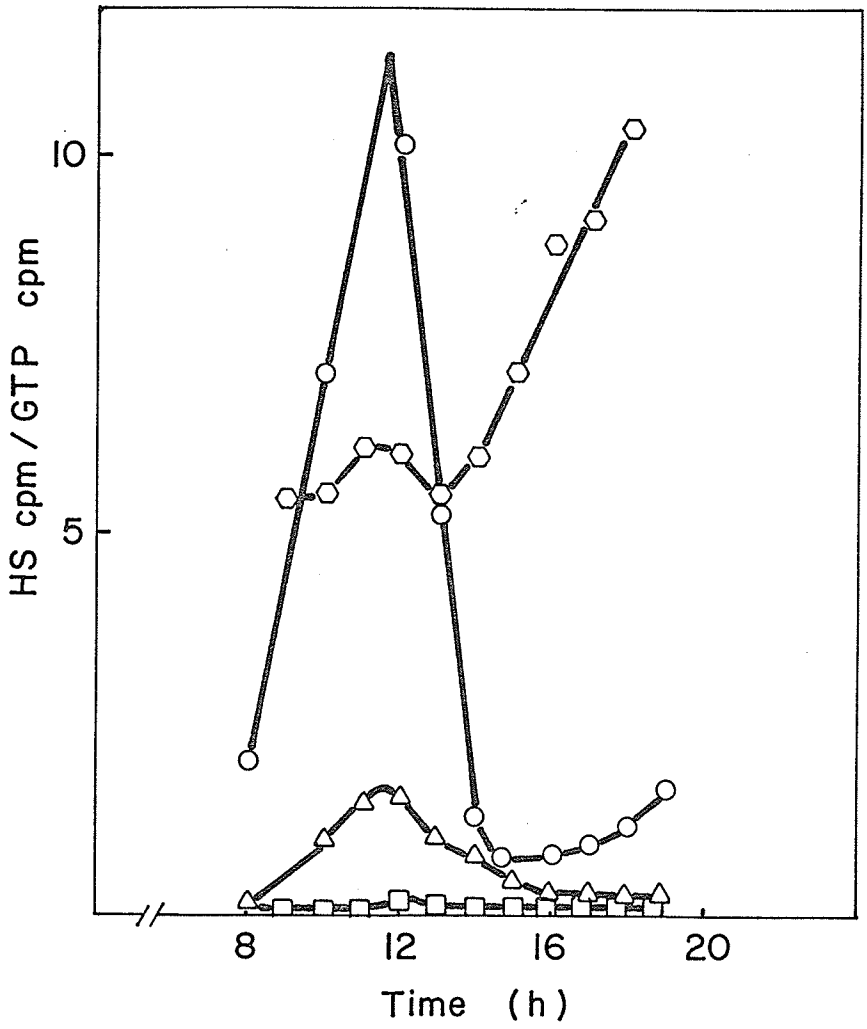

HS cpm x10⁻⁴ / 25 ml sample
mg dry wt / 25 ml sample
Time (h)
1
2
3
4
5
2
4
6
8
10
6
10
14
18

Figure 6: The relative pool sizes of HS compounds measured at hourly intervals over the last 12 h of the growth cycle. Three litre carboy culture was supplemented with ^{32}P-orthophosphate (described in Methods). At hourly intervals, 25 ml of culture was filtered and the cells extracted with 1M formic acid. ^{32}P-radioactivity in HS compounds, ATP and GTP was determined as described in Methods. HS3/GTP (O), HS2/GTP (Δ), HS1/GTP (□) and ATP/GTP (⬡).

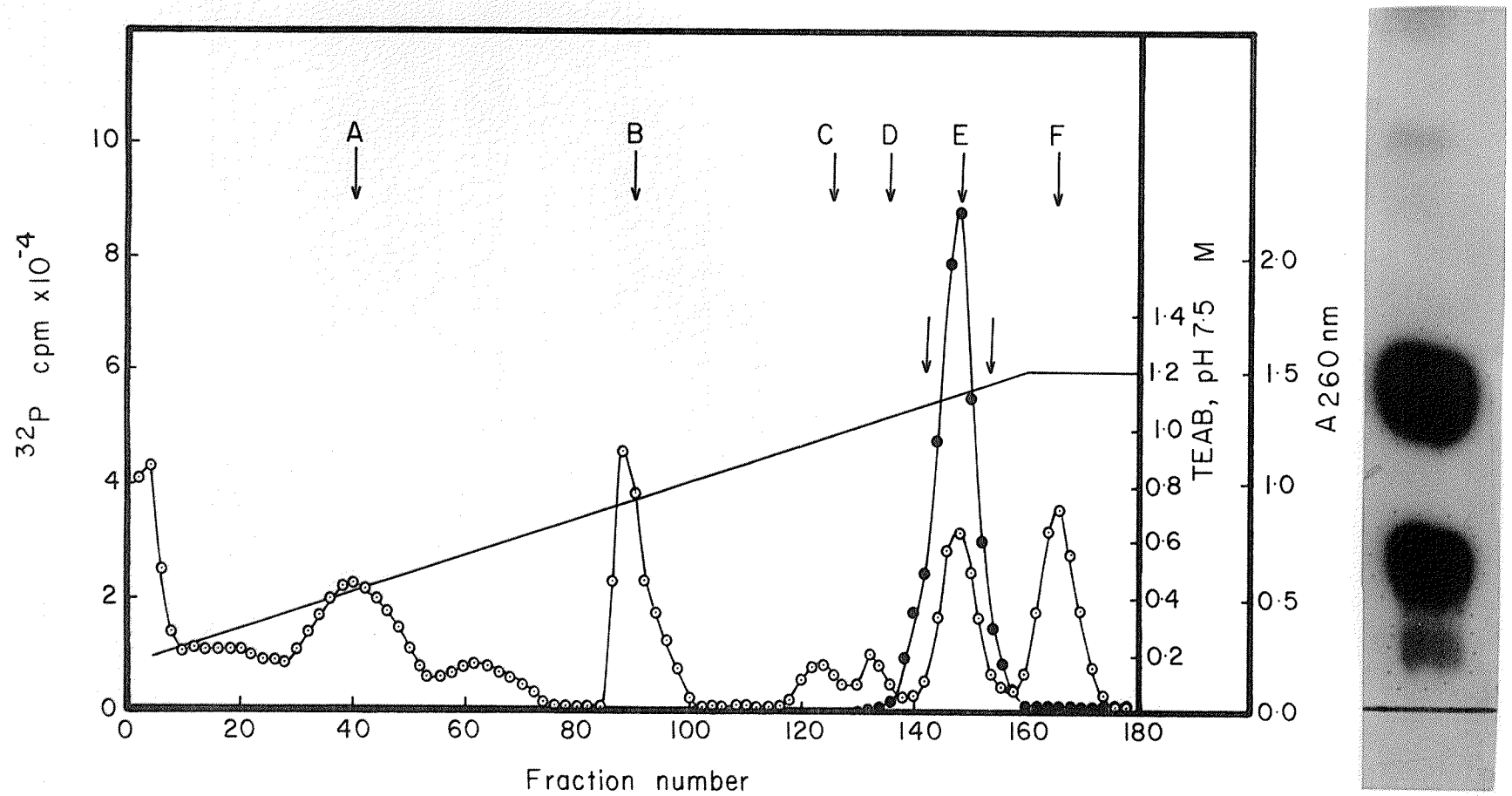

10
5
HS cpm / GTP cpm
8
12
16
20
Time (h)

The HS compounds, as well as other acid soluble nucleotides, were extracted from the cells with 1M formic acid (1ml acid per gram wet weight of mycelia) for 1 h at 0^{o}. The acid was recovered after removing the cells by suction filtration, and the filtrate carefully adjusted to neutrality with ammonium hydroxide. The filtrate was then diluted 10-fold with deionized water before fractionating on Sephadex-A25.

Figure 7 shows the separation at pH 8 of unlabelled extract, to which partially purified ^{32}P labelled HS compounds (see Methods) were added. All the ^{32}P radioactivity eluted from the column at approximately 1.1 M TEAB buffer, and was coincident with the A_{260} activity of fractions labelled E. A fraction in this peak was evaporated to dryness under vacuum, washed several times with deionized water, redissolved in a small volume of water and an aliquot chromatographed on PEI-cellulose in solvent 1. An autoradiogram of this is shown in figure 7 (right panel), which indicates that all three HS compounds eluted from the column at the same molarity of TEAB buffer.

For routine isolation of HS compounds we used unlabelled acid extract mixed with ^{32}P labelled acid extract prepared from a small scale culture

Figure 7: Chromatography of cold formic acid extract from 12-13 h old Achlya mycelia to which was added partially purified ^{32}P labelled HS compounds (see Materials and Methods) in a column (3.0 x 45 cm) of DEAE-Sephadex preequilibrated with 0.2M TEAB buffer, pH 8. After the extract was loaded on the column, it was washed with 500 ml 0.2M TEAB buffer and then nucleotides were eluted with 500 ml of a linear (0.2-1.2M) gradient of TEAB buffer. Four ml fractions were collected. The A_{260} value (⊙) of each fraction was determined and the ^{32}P content (●) estimated by the cerenkov technique. Also shown is an autoradiogram of Peak E constituents labelled with ^{32}P.


A
B
C
D
E
F
^{32}P cpm x10^{-4}
TEAB, pH 7·5 M
A 260 nm
Fraction number
0
2
4
6
8
10
0·2
0·4
0·6
0·8
1·0
1·2
1·4
0·0
0·5
1·0
1·5
2·0
0
20
40
60
80
100
120
140
160
180

(see Methods). This was chromatographed on Sephadex-A25 and the results are shown in figure 8. The constituents of peak E were analyzed by PEI chromatography and the results are shown in the autoradiogram in figure 8 (right panel). As was expected all three HS compounds eluted at approximately 1.1M TEAB buffer. The autoradiogram also shows other ^{32}P labelled compounds present in peak E. Peak F was found to contain mainly ATP.

Peak E material (Fig. 8, as shown by the arrows) was pooled, evaporated to dryness, washed with water, resuspended in 0.05M formate buffer pH 3.6 and chromatographed on Sephadex-A25 using a sodium chloride gradient. Normally peak E material from three different column separations were mixed with peak E material containing radioactive ^{32}P-HS compounds. The results are shown in figure 9. Four peaks are discernible, each showing uv-light absorption at 260 nm and ^{32}P-radioactivity. ATP which eluted off the column at approximately 0.21 M NaCl, arose from cross contamination from peak F material. HS3 was routinely eluted from the column between 0.24-0.28 M NaCl; HS2 eluted between 0.3-0.34 M NaCl and HS1 between 0.38-0.4 M NaCl. Each peak was found to contain a

Figure 8: Chromatography of ^{32}P-labelled formic acid extract of 12-13 h old Achlya mycelia in a column (3.0 x 45 cm) of DEAE-Sephadex preequilibrated with 0.2M TEAB buffer pH 8.0. After the extract was loaded, the column was washed with 500 ml 0.2M TEAB buffer and then nucleotides were eluted with 500 ml of a linear (0.2-1.2M) gradient of TEAB buffer. Four ml fractions were collected. The A_{260} value (⊙) of each fraction was determined and the ^{32}P content (●) estimated by the cerenkov technique. Also shown is an autoradiogram of the constituents in peak E.

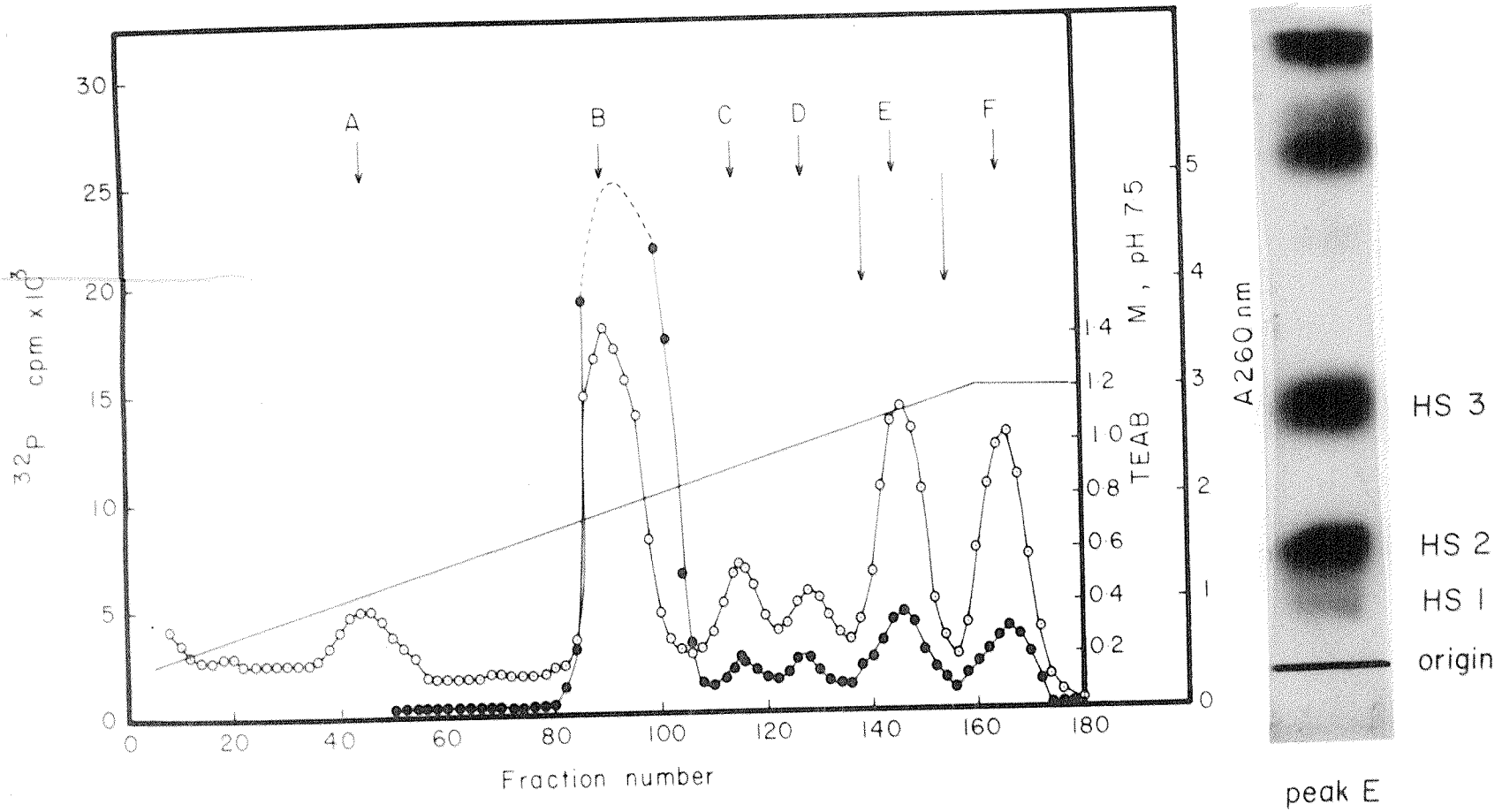
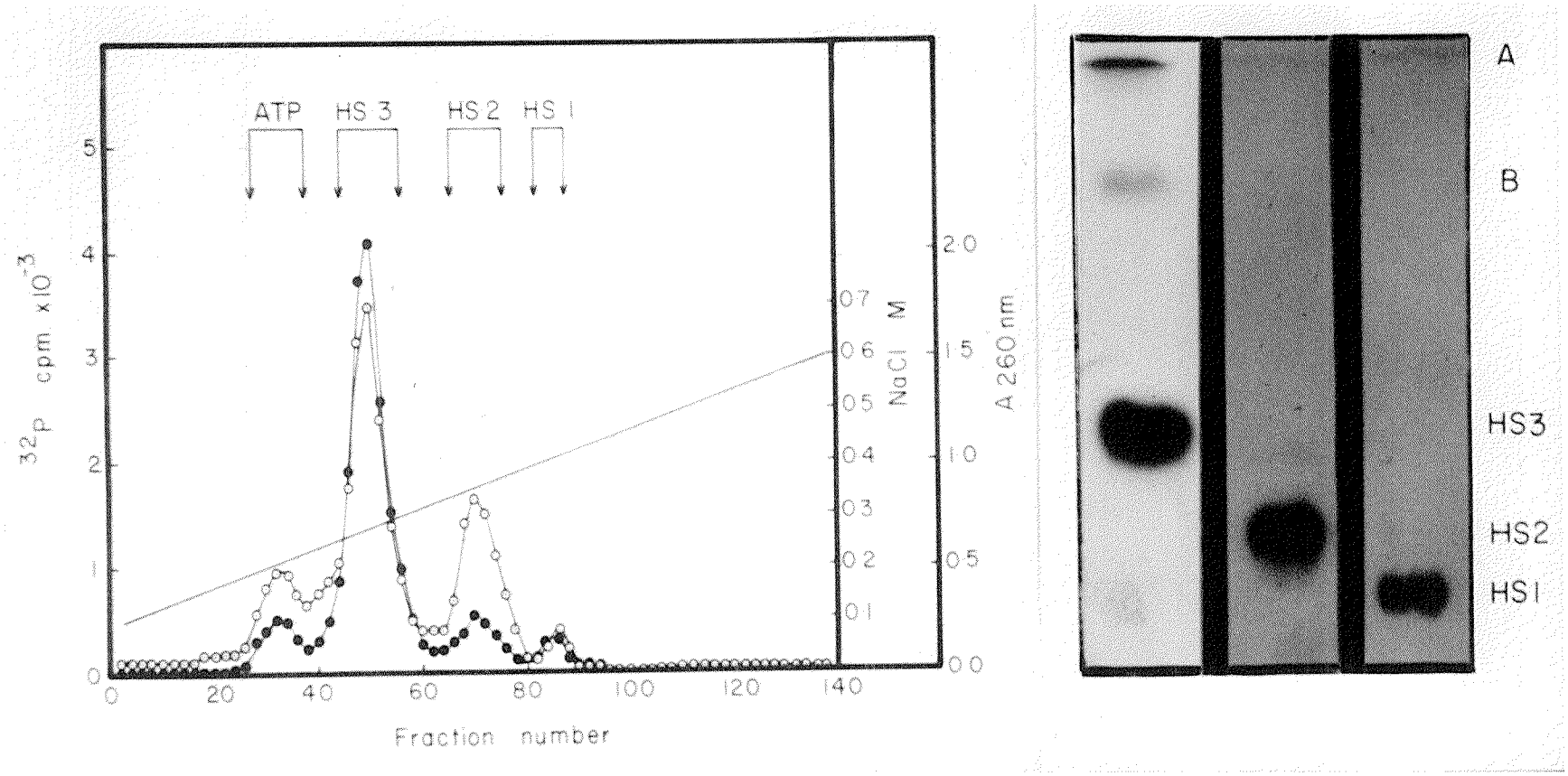

A
B
C
D
E
F
30
25
20
15
10
5
0
^{32}P cpm x10^{3}
0
20
40
60
80
100
120
140
160
180
Fraction number
1.4
1.2
1.0
0.8
0.6
0.4
0.2
M, pH 7.5
TEAB
5
4
3
2
1
0
A260nm
HS 3
HS 2
HS 1
origin
peak E

Figure 9: Sephadex-A25 chromatography of the pooled fractions of Peak E from figure 8. Material was loaded on a column (1.0 x 30 cm) which was then flushed with 250 ml 0.1M NaCl: 0.05M Na formate buffer, pH 3.6. The nucleotides were eluted with a linear (0.1-0.6M) gradient of NaCl in 0.05M Na formate pH 3.6. Four ml fractions were collected and the A_{260} (⊙) and ^{32}P content (●) determined. Arrows indicate fractions that were pooled. Panel on right shows autoradiograms of the contents of the last three peaks chromatographed on PEI-cellulose with solvent I. Breakdown products of HS3 arising from chromatography on PEI-cellulose, are evident in the first of the three autoradiographic panels. They are marked A and B and they migrate in areas corresponding with authentic UDP and ADP respectively.


ATP
HS 3
HS 2
HS 1
^{32}P cpm x 10^{-3}
NaCl M
A 260 nm
Fraction number
A
B
HS3
HS2
HS1

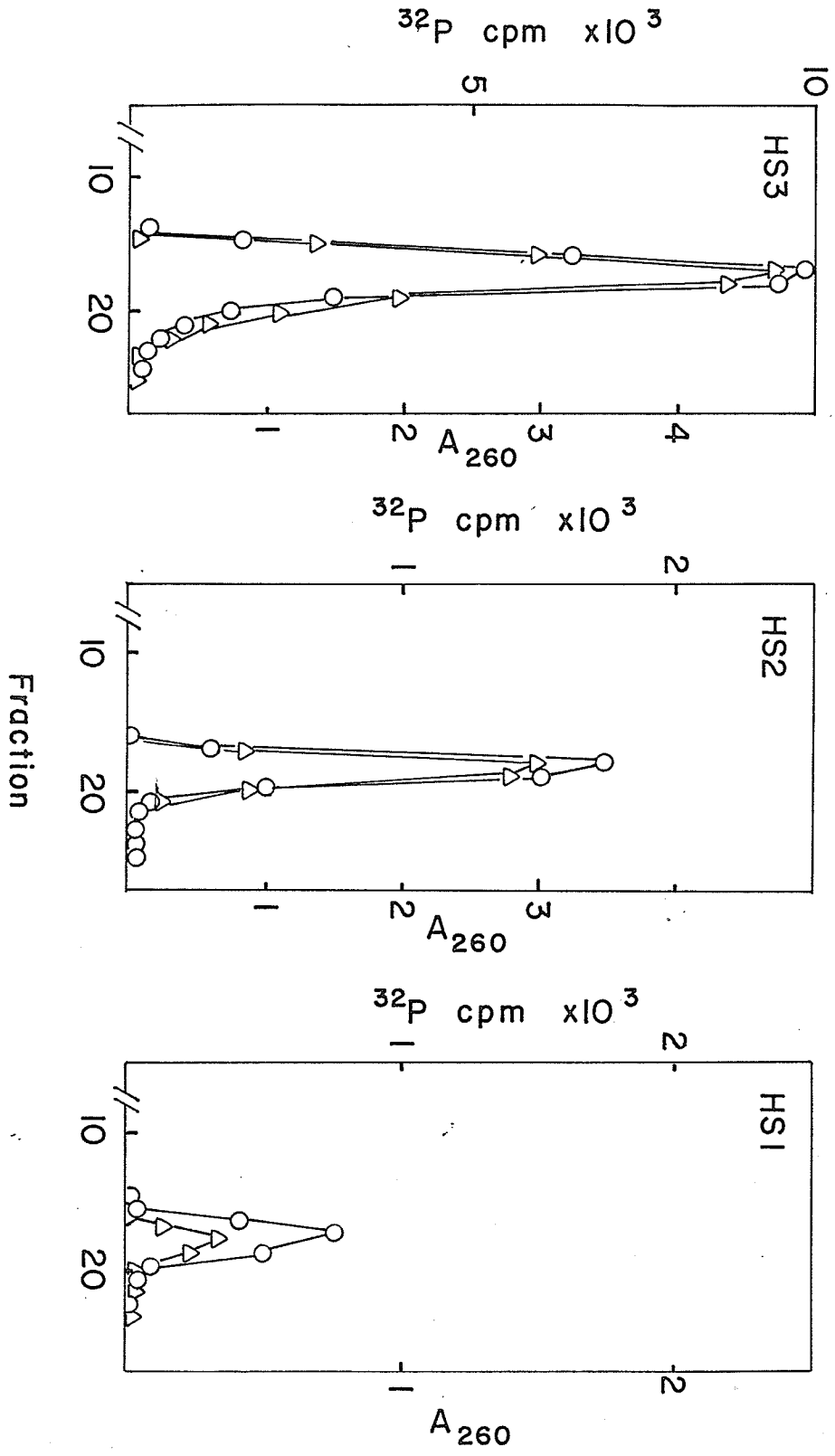
single component after PEI-cellulose chromatography and autoradiography as shown in figure 9 (right panel).

The fractions within the boundaries of the arrows of figure 8 were pooled separately, the pH adjusted to 7.5 with sodium hydroxide, concentrated via evapomix and desalted on Sephadex-G10 (see fig. 10). The fractions containing desalted HS compounds were pooled separately, concentrated to dryness under vacuum at 30^{o}, washed several times with water, and redissolved in a known volume of water. These were stored at -20^{o} for further use.

Each preparation of HS1, 2 and 3 was shown to contain a single component after PEI-cellulose chromatography (see right panel fig. 9) suggesting that each was free from contaiminating nucleotides.

The purification procedure described above was reproducible, although considerable loss of the HS compounds during the isolation was not uncommon. The reasons for this are unclear but a number of possibilities seem quite likely. These are (1) presence of specific or non specific enzymes in the acid extract which could degrade any or all of the HS compounds; (2) an inherent physical

Figure 10: Sephadex-G10 chromatography of the pooled (and concentrated) fractions containing HS compounds from figure 9. For details see Methods. Fractions were analyzed for ^{32}P-radioactivity (O) and A_{260} (Δ).


^{32}P cpm x10^{3}
HS3
HS2
HS1
A$_{260}$
Fraction

lability of the compounds to the isolation procedure. The relative stability of each of the purified HS compound will be discussed later.

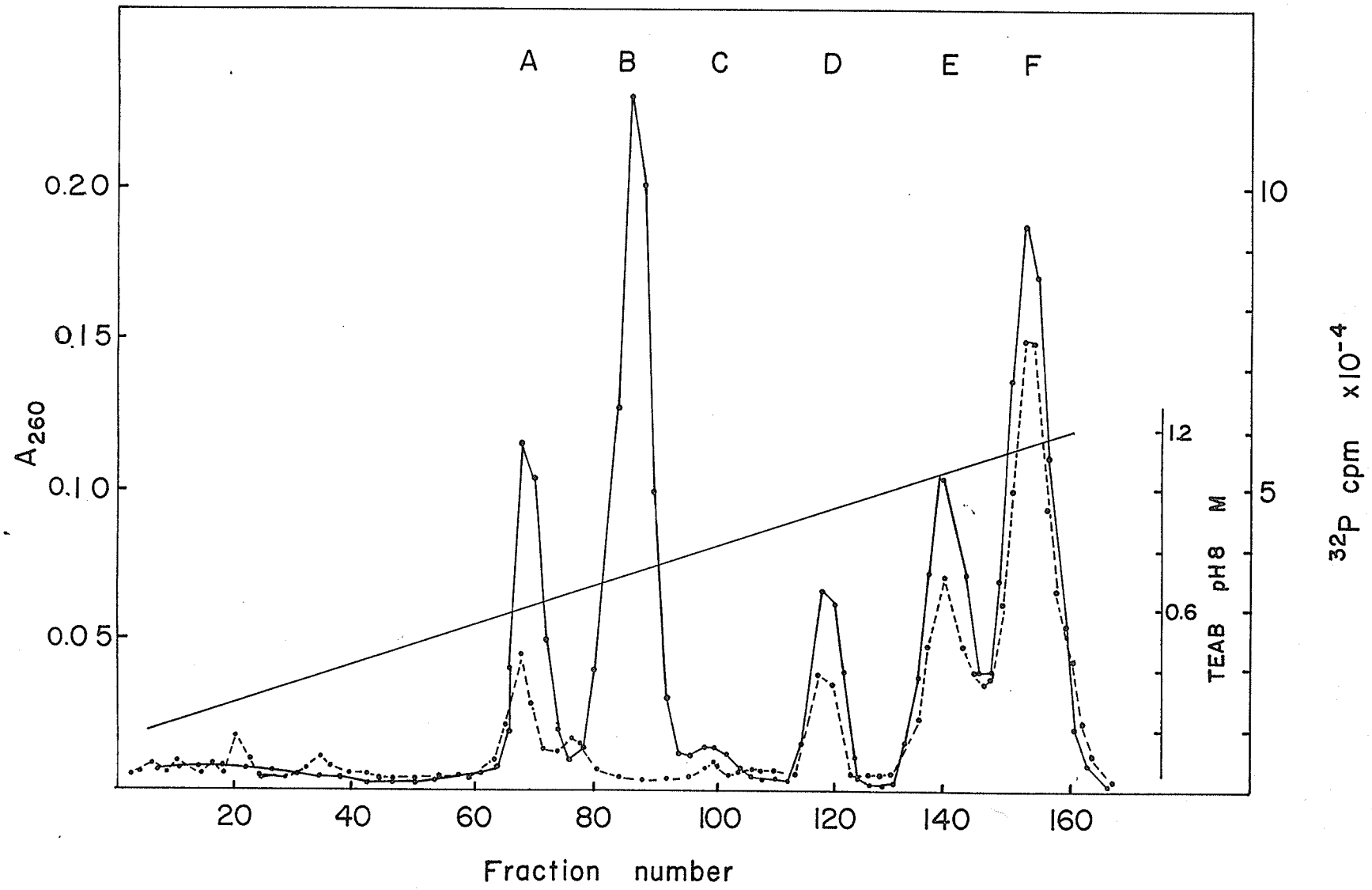
The quantity of HS compounds isolated from Achlya varied from preparation to preparation. HS compounds were quantitated using the standard orcinol procedure for ribose determination or absorbance at 260 nm. The quantity of HS compounds isolated from 100-200g (wet weight) of Achlya mycelia ranged from 1-10 μmoles for HS3; 0.1-2 μmoles HS2 and 0.03-0.25 μmoles HS1.

2. from Chinese Hamster Ovary Cells

Chinese hamster ovary cells were routinely cultured as described by Goh and LéJohn (1977). Mammalian cell HS3 was isolated from CHO cells grown in 16 ounce Brockway bottles (as described by Lewis et al. 1977). Cells were plated into 20 bottles and allowed 24 h at 37^{o} to reach cell densities of $1\text{-}2 \times 10^{7}$ cells per bottle. The medium was then removed and the cells were washed two times with warm medium lacking glutamine and finally incubated at 37^{o} in α-minimal medium minus glutamine plus 10% dialyzed fetal calf serum. One Brockway bottle was supplemented with 1 mCi ^{32}P-orthophosphate. After 4 h, the medium was poured off, the cells were washed once with phosphate buffered saline and extracted with 1.0M formic acid. The formic acid extract was removed by pipette and centrifuged to remove cells and debris. HS3 was then isolated and purified from the formic acid extract as described for Achlya HS compounds.

Chromatography of the labelled extract on Sephadex-A25 at pH 8.0 is shown in figure 11. Mammalian cell HS3 eluted from the column at

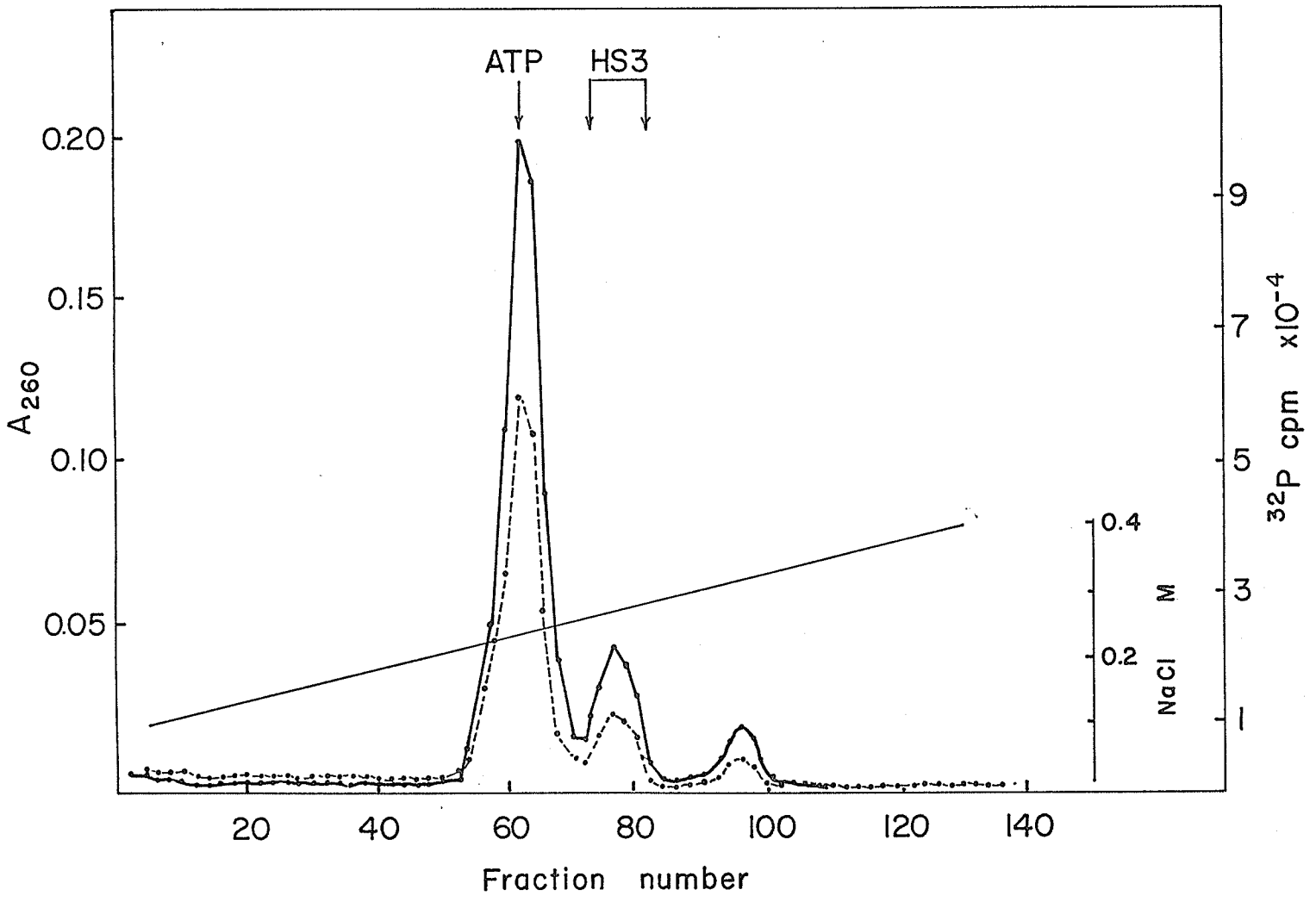
Figure 11: Chromatography of ^{32}P-labelled formic acid extract of Chinese hamster ovary cells, starved of L-glutamine for 4 h , in a column (3.0 x 45cm) of DEAE-Sephadex preequilibrated with 0.2M TEAB buffer pH 8. After the extract was loaded, the column was washed with 500ml 0.2M TEAB buffer and then nucleotides were eluted with 500ml of a linear (0.2-1.2M) gradient of TEAB buffer. Four ml fractions were collected. The A_{260} value (•---•) of each fraction was determined and the ^{32}P content (•—•) estimated by the cerenkov technique.


A
B
C
D
E
F
0.20
0.15
0.10
0.05
A_{260}
10
5
^{32}P cpm $\times 10^{-4}$
1.2
0.6
TEAB pH8 M
20
40
60
80
100
120
140
160
Fraction number

approximatley 1.0M TEAB buffer in the peak designated E (figure 11). The peak labelled F contained material that comigrated with ATP. Material from peak E and peak F (used as a marker) were chromatographed on Sephadex-A25 using a NaCl gradient in 50mM formate buffer pH 3.6. This is shown in figure 12. The mammalian cell HS3 eluted from the column at approximately 0.27M NaCl. The fractions containing mammalian cell HS3 were collected and desalted. Mammalian cell HS3 was stored at -20^{o}for further use.

The quantity of mammalian HS3 isolable by this method was in the range 0.1-0.3 μmoles. This is based on the ribose content as estimated by the standard orcinol procedure. This amount is relatively small compared to that obtained from Achlya; thus HS3 from Achlya was used primarily for analysis. However, mammalian HS3 was analyzed for various components found in Achlya HS3.

Figure 12: Sephadex-A25 chromatography (column 1x30cm) of the pooled fractions of peak E and F from figure 11, using a linear (0.1-0.4M) gradient of NaCl in 50mM formate pH3.6 buffer. Four ml fractions were collected and the A_{260} (o---o) and ^{32}P content (o—o) estimated. Arrows indicate fractions containing mammalian HS3 that were pooled and desalted as described for Achlya HS3.


ATP
HS3
0.20
0.15
0.10
0.05
A_{260}
0.4
0.2
M
NaCl
9
7
5
3
1
^{32}P cpm $\times 10^{-4}$
20
40
60
80
100
120
140
Fraction number

C. ACHLYA HS3: PHYSICAL, CHEMICAL AND ENZYMATIC ANALYSIS

1. Spectral Analysis

Purified Achlya HS3 was spectrally analyzed between 200 and 300 nm at pH 1, 7 and 12 and the data are summarized in table 2. The spectral data of adenosine and uridine are also included (as HS3 contains adenosine and uridine: see "Purine and Pyrimidrine Analysis"). The uv-spectrum of HS3 at pH 7 is shown in figure 13. HS3 absorbs maximally around 260-262 nm at each pH value with the wavelength of minimum absorption increasing slightly as the pH changed from 1 to 12. This behaviour is characteristic of uracil and uracil containing nucleotides. The wavelengths of maximal and minimal absorption, and the spectral ratios are similar to those of adenosine and uridine.

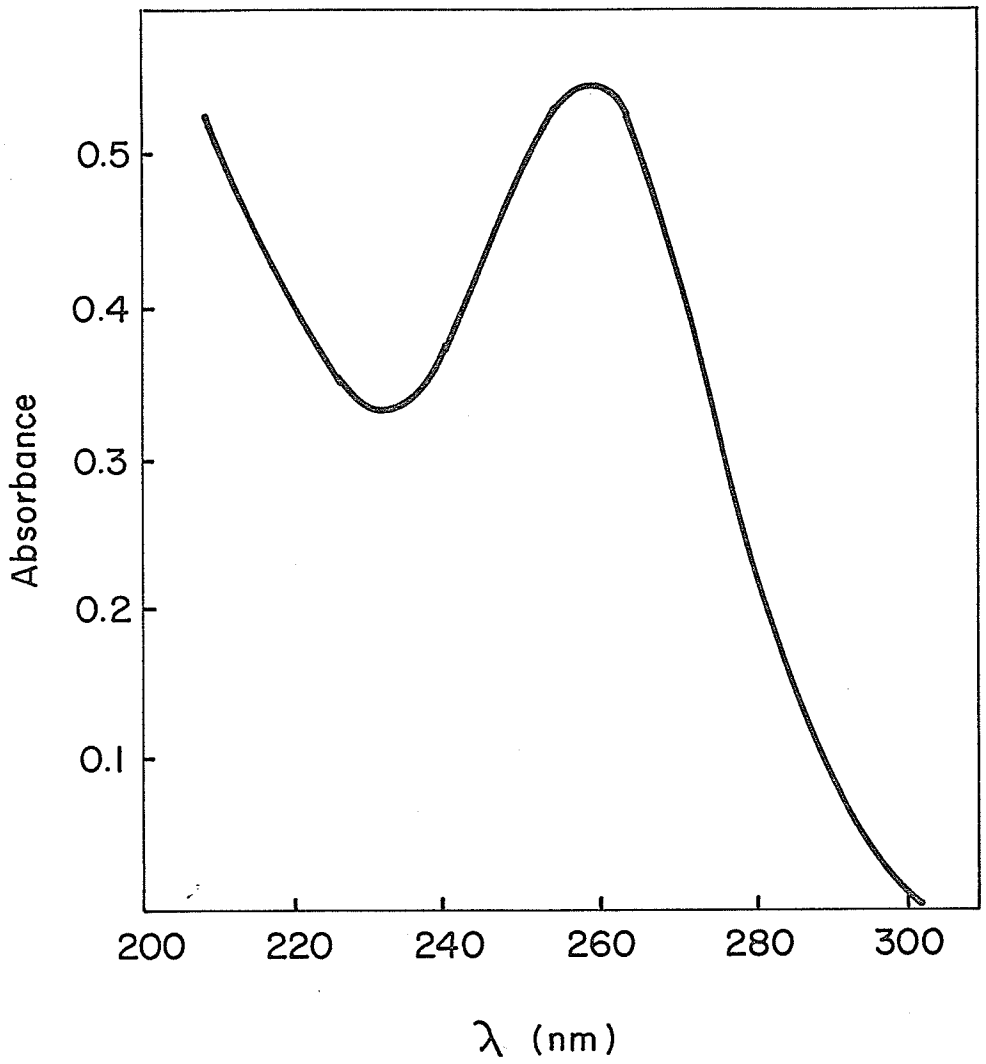
HS3 absorbs maximally in distilled water (pH 5). The extinction coefficient of HS3 at 260 nm (H_2O) was calculated as 14,500 ± 500. This was based on the measurable purine bound ribose (see "Carbohydrate Analysis") of which there is one molecule per HS3.

Table 2 Spectral Data of HS3 and Standards

compound	pH	λ nm max	λ nm min	Absorbance ratios 250/260	280/260	290/260
HS3	1	262	232	0.77	0.56	0.31
	7	260	232	0.84	0.45	0.16
	12	260	235	0.86	0.39	0.10
Adenosine*	1-7	257	230	0.84	0.21	0.03
	12	259	227	0.78	0.14	0.002
Uridine*	1-7	262	231	0.74	0.35	0.03
	12	262	242	0.83	0.29	0.02

*data for standards taken from Burton (1969).

Figure 13: Ultraviolet absorption spectrum of HS3 in 0.01 M Na phosphate buffer pH 7.0.


Absorbance
0.5
0.4
0.3
0.2
0.1
200
220
240
260
280
300
λ (nm)

There was a decrease in the molar absorptivity of HS3 as the salt concentration (ionic strength) of the solution increased. This is shown in table 3. Also shown in table 3 are data obtained for commercial nucleotides. HS3 lost 35-40% of its absorbance at 260 nm upon addition of 1.0M phosphate pH 7 buffer, while higher salt concentrations did not cause any further decrease. Data obtained for common nucleotides reveals that only UTP displayed a decrease in absorbance with increasing salt concentrations while CTP was unaffected and ATP only mildly affected at very high salt concentrations. This appears to be a characteristic of uracil containing nucleotides and polynucleotides as demonstrated by Simkins and Richards (1967). They have attributed this effect to base stacking complications.

The uv-light absorbance of HS3 at 260 nm showed a hyperchromic effect of 10% during incubation with 0.33N KOH at 37^{o} for 30 min. An increase of 20-25% in the uv-light absorbance at 260 nm was observed during treatment of HS3 with 1N HCl for 30 min at 100^{o}, a treatment which degrades the molecule.

These data suggest that the ultraviolet absorption property of HS3 is influenced by salts and the integrity of the molecule.

Table 3. Effect of increasing salt concentration on the absorption at 260 nm of HS3 and authentic nucleotides

KH_2PO_4 at pH 7.5; M	% change at A_{260} HS3	UTP	CTP	ATP
0	0	0	0	0
0.01	-24	-19	0	0
0.10	-27	-27	0	0
0.50	-35	-30	0	-4
1.0	-39	-34	0	-6

-, signifies a reduction in absorbance.

2. Purine and Pyrimidine Analysis

^{32}P-labelled HS3 was acid hydrolyzed *in vacuo* for 1 h and 20 h in 1N HCl at 105°. The products were chromatographed on paper in solvent III, and on thin layer cellulose plates in solvent IV. After 1 h hydrolysis, HS3 was degraded into two uv-light absorbing entities designated a and c in table 4. Component a had an R_f of 0.13 in solvent III and 0.71 in solvent IV. This compared to adenine, R_f 0.12 in solvent III and 0.74 in solvent IV. Component c migrated with R_f of 0.72 in solvent III and 0.10 in solvent IV, which compared to UMP R_f 0.70 in solvent III; 0.12 in solvent IV. Component a did not contain ^{32}P-label where as c did. Both products were eluted from the paper, desalted on Sephadex-G10 and the uv spectra determined at pH 7.0. The spectra for a and c were similar to those of adenine and uridine monophosphate respectively (table 5). Molar absorptivities at 260 nm of 13.3×10^3 for adenine and 9.9×10^3 for UMP were used to compute the mole ratio of 1:1 for the products a and c (table 5). After 24 h acid hydrolysis of HS3, two products were observed; a uv-absorbing product designated b

TABLE 4. Chromatographic separation of the uv-light absorbing products of HS3 after hydrolysis in 1N HCl.

HS3 product	Hydrolysis time (h)		R_f	R_f
	1	20	solvent III	solvent IV
a	+	+	0.13	0.71
b	-	+	0.54	0.43
c	+	-	0.72	0.10
uridine*			0.56	0.41
UMP*			0.70	0.12
adenine*			0.12	0.74

+ indicates observed uv-light absorbing product

* standards

TABLE 5. Spectral analysis at pH 7.0 of uv-light absorbing products (from table 4) of HS3 after 1 h hydrolysis in 1N HCl

product	λ nm max	λ nm min	Absorbance ratios 250/260	280/260	290/260	nmoles*
a	258	228	0.90	0.17	0.03	52
c	263	230	0.86	0.36	0.02	53
adenine	260	229	0.76	0.13	0.005	
UMP	260	231	0.74	0.39	0.03	

* Extinction coefficients of 13.3×10^3 and 9.9×10^3 were used to calculate amounts of products a and c respectively.

in table 4 migrated with uridine (R_f 0.56 in solvent III and R_f 0.43 in solvent IV) while a fluoresent product migrated with R_f 0.13 in solvent III and 0.71 in solvent IV which was probably adenine.

The base constituents of ^{32}P-labelled HS3 were also determined after digestion of the molecule by a combination of enzymes. HS3 was digested with a mixture of nucleotide pyrophosphatase and bacterial alkaline phosphatase for 2 h (see Methods). The hydrolysis products were separated chromatographically in two dimensions on thin layer cellulose using solvents IV and V. Authentic adenosine and uridine were used as markers. As shown in table 6, HS3 contained two uv-absorbing entities which comigrated in both dimensions with adenosine (product A) and uridine (product B). Products A and B were eluted from the plate and their absorption at 260 nm was measured. Molar absorptivities at 260 nm of 14.3×10^3 (for adenosine) and 9.9×10^3 (for uridine) were used to compute a mole ratio of 1:1 for products A and B (Table 6). The carbohydrate content of products A and B (after elution from the cellulose with water)

TABLE 6. Chromatographic properties of the products derived from HS3 degraded by bacterial alkaline phosphatase and nucleotide pyrophosphatase.

HS3 product	R_f solvent IV	R_f solvent V	nmoles **
A	0.63	0.30	48
B	0.38	0.76	45
adenosine*	0.64	0.31	
uridine*	0.38	0.74	

Enzymes used at 1μg/A_{260} of compound in 0.2M tris-HCl, pH 7.5 and 0.2M $MgCl_2$. Mixtures incubated for 2 h at 37°.

* authentic adenosine and uridine

** Extinction coefficients of 14.3×10^3 and 9.9×10^3 were used to calculate amounts of products A and B respectively.

was determined as described in "Carbohydrate Analysis".

These results indicate that acid hydrolysis (1N HCl) for 1 h releases adenine and uridine (in some cases a small amount of uracil was also observed). The mole ratio of these two components is 1:1. HS3 (approximately 1 O.D. unit) digested enzymatically released products which comigrated with adenosine and uridine in two dimensions. It was concluded that the uv-light absorbing components of HS3 were adenosine and uridine.

The 260 nm (H_2O) extinction coefficient of HS3 has been calculated to be $14.5 \times 10^3 \pm 0.5 \times 10^3$. The sum of the extinction coefficients of adenosine and uridine is 23.2×10^3 (Burton 1969). This indicates that the uv-light absorbing components of HS3 (adenosine and uridine) do not absorb maximally in the intact molecule. Degradation of HS3 by 1N HCl (30 min at 105°) causes a 25% increase in uv-light absorptivity of HS3, suggesting that the reason for a low extinction coefficient is probably due to the molecular configuration of the molecule.

3. Amino Acid Analysis

After acid (6N HCl) hydrolysis of HS3 *in vacuo* at 105^{o} for 20 h, analysis for amino acids was carried out. After two dimensional thin layer chromatography with solvents VI and VII, ninhydrin spray revealed a single major red-orange spot (figure 14) which was coincident with authentic glutamate (R_f 0.40 and 0.48). The presence of glutamate was confirmed and quantitated using a Beckman model 121 amino acid analyser which showed that there was one mole of glutamate per mole of ribose (table 7). Two other amino acids, aspartic acid and glycine, were detected by the amino acid analyser but they were present to the extent of only 9% and 11% respectively of the glutamate content. Either they were contaminants of the preparation or they were degradation products from adenine and uracil present in HS3. Adenine was shown to yield glycine upon acid hydrolysis.

The glutamate released from HS3 was analysed for the optical form using D or L amino acid oxidase (see Table 8). Glutamate was hydrolyzed to α-keto-glutarate by D-amino acid oxidase but not by L-amino acid oxidase indicating that the amino acid released

Figure 14: Photographic record of a chromatogram prepared during amino acid analysis of HS3 using two dimensional chromatography on cellulose thin layer plates. First dimension developed in solvent VI and second dimension in solvent VII. Amino acids located by spraying with 3% ninhydrin solution in ethanol.

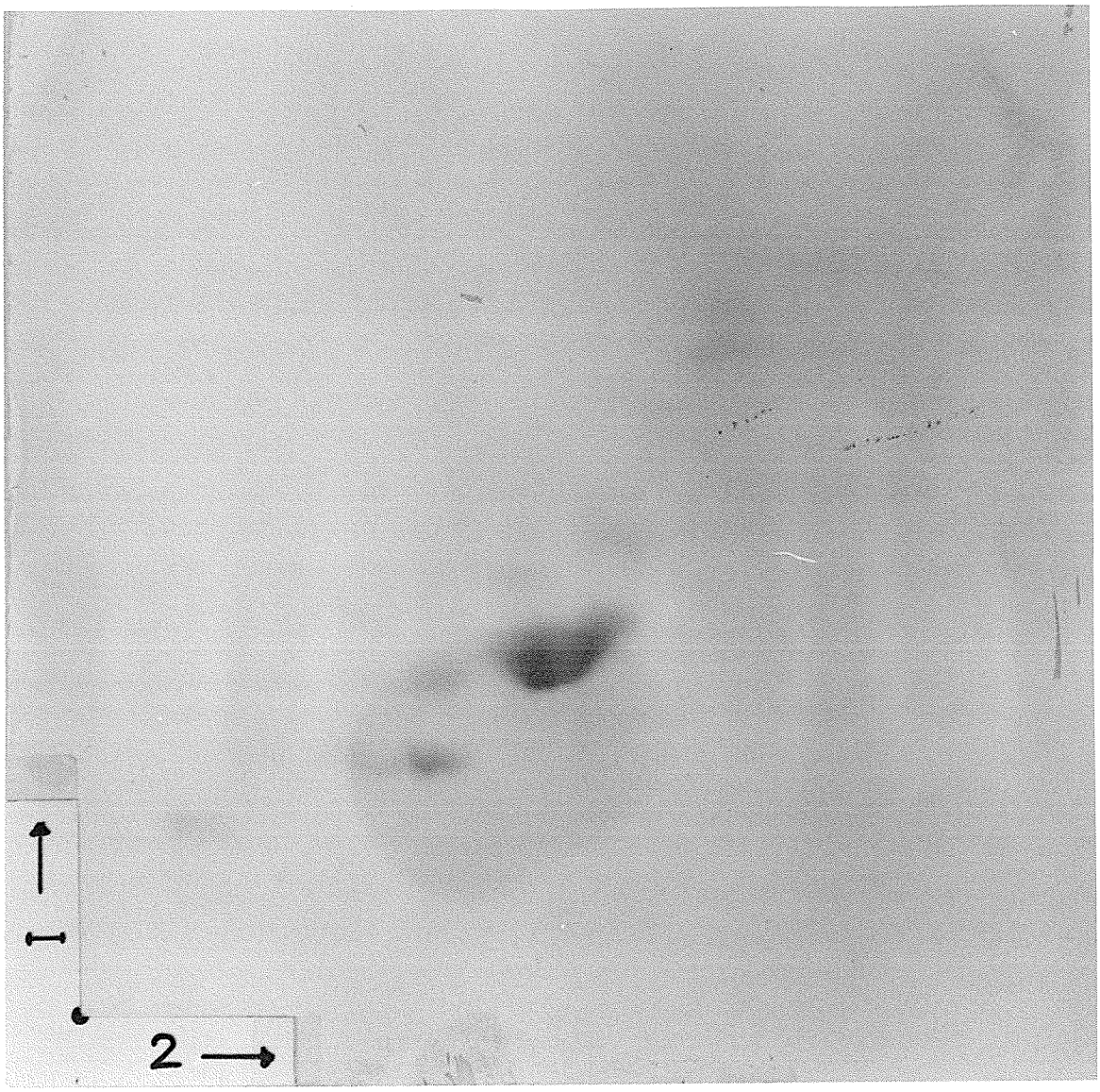

1
2

TABLE 7. Stoichiometry of HS3 components

compound	nmoles				Ratios			
	ribose	PO_4	base*	glu	ribose/ base	base/ PO_4	ribose/ PO_4	ribose/ glu
HS3	10	76	18	9	1:1.8	1:4.2	1:7.6	1:1.1
ATP	8	25	8	-	1:1	1:3.1	1:3.1	-

* Total base was calculated from A_{260} values of acid hydrolyzed products with an extinction coefficient 11.5 x 10^3 per base.

TABLE 8. Analysis of the isomeric form of glutamate (released from HS3 by acid hydrolysis*) using D or L amino acid oxidase.

compound	D amino acid oxidase	L amino acid oxidase
D-glutamate	-	+
glutamate (HS3)	-	+

*Acid hydrolysate of HS3 (3 units at 260nm) evaporated to dryness (resuspended in water and evaporated to dryness several times to remove acid) and brought to]00 µl with 0.]M citrate buffer, pH 6.0. Equivalent aliquots were analyzed for the isomeric form of glutamate as described in Methods.

+ indicates glutamate observed

- indicates abscence of glutamate (deaminated to ∝ketoglutarate by the amino acid oxidase).

from HS3 by acid hydrolysis is the D-optical isomer.

Glutamate was also released from HS3 using 1N HCl hydrolysis for 20 h at 105°. Two dimensional chromatography of such a mixture revealed the presence of another ninhydrin reacting compound which migrated behind glutamate in both dimensions. This component is considered an amino sugar and will be discussed in a later section. Glutamate was also released from HS3 after treatment with 0.5N KOH at 105° for 2 h.

Glutamate was not released from HS3 after heat treatment in water (20 h in vacuo at 105°), 1 h acid hydrolysis (1N HCl), KOH treatment (0.3N for 20 h at 37°), periodic acid oxidation (Cashel and Kalbacher 1970) or after digestion of HS3 with a mixture of bacterial alkaline phosphatase and nucleotide pyrophosphatase. The significance of these results will be discussed.

4. Carbohydrate Analysis

The purine-bound ribose content of HS3 was determined by the orcinol procedure (Brown 1946). The results shown in table 7 indicate that HS3 contained only one purine-bound ribose per molecule.

For the estimation of pyrimidine-bound ribose the procedure of Haavaldesen et al. (1959) was used. This procedure requires that the sample be pretreated with 1% sodium amalgam for 4-8 hours at room temperature before the orcinol reaction. Reduction of pyrimidines by sodium amalgam is thought to destabilize the linkage between the sugar and the nitrogen base allowing cleavage by the acid in the orcinol reagent. As is shown in table 9 this treatment of HS3 did not result in any additional pyrimidine-bound ribose. However, acid hydrolyzed HS3, treated with sodium amalgam showed a 30% increase of estimable ribose (48 moles) over the non-sodium amalgam treated control (table 9).

The separated nucleosides produced by enzyme digestion of HS3 (products A and B, table 6) after elution from the cellulose plate with water, were

TABLE 9. Analysis of purine and pyrimidine-bound ribose content of HS3 by orcinol with and without 1% sodium amalgam pretreatment

compound	nmoles recovered without	with
HS3	37	36
*acid hydrolyzed HS3	36	48
UTP (10 nmoles)	-	10
ATP (20 nmoles)	20	19
*acid hydrolyzed ATP	19.5	5.2
**Adenosine (product A)	46	-
**Uridine (product B)	-	50

* hydrolyzed for 1 h in 1N HCl *in vacuo*

** Two dimensionally separated components from enzyme digest of HS3 (table 6) eluted from cellulose.

analysed for ribose. Table 9 shows that the uridine nucleoside (product B) was found to contain pyrimidine-bound ribose equivalent in amount to the purine-bound ribose in adenosine (product A), suggesting that HS3 contains 2 moles of ribose per molecule, one purine-bound and one pyrimidine-bound. The orcinol assay of HS3 only estimated the adenine-bound ribose and sodium amalgam reduction of HS3 did not result in the estimation of the pyrimidine-bound ribose.

Kochetkov et al. (1964, 1972) have reported that the hydrogenation of uridine derivatives by sodium amalgam is considerably retarded in the presence of adenosine or in the case of uridine dinucleotide sugars. They have suggested that this may be the result of the intramolecular interaction between the heterocyclic base and sugar residue in the nucleoside diphospho-carbohydrate molecule. Since HS3 contains adenosine and also a uridine diphospho-sugar moiety (described in a following section) the inability to measure the uridine bound ribose in HS3 by reduction is probably due to interference by the presence of these components.

The acid hydrolyzed sample of HS3, did show a 30% increase in ribose after pretreatment with

sodium amalgam. One hour acid hydrolysis of HS3 releases UMP, adenine and ribose (phosphate) as shown in table 5. However, the degradation of HS3 and sodium amalgam treatment still did not result in recovery of the expected amount of purine and pyrimidine-bound ribose (ie twice that of purine-bound ribose or 72 nmoles). The value obtained (48 nmoles) was approximately 66% of the expected amount. The reason for this could be due to either interference, by other components in the hydrolyzate, in the reduction of the uridine components or an inability to measure the acid released purine-bound ribose (after sodium amalgam reduction) by the orcinol technique. The results obtained for authentic ATP would support the latter hypothesis. As is presented in table 9 only 25% (5.2 nmoles) of the expected amount (20 nmoles) of ATP was estimable by orcinol following Na-amalgam reduction as compared to direct acid hydrolysis. This amounts to a 75% loss of estimable purine-bound ribose. This is caused by sodium amalgam reduction of the free ribose phosphate (released from the adenosine moiety by acid) into

ribitol (Pigman and Goepp 1948). Ribitol is not reactive with orcinol reagent. Assuming only 25% of the purine-bound ribose and 100% of the pyrimidine-bound ribose of HS3 was estimated after acid hydrolysis and reduction, a value of 45 nmoles would be expected. This agrees with the value of 48 nmoles obtained.

HS3 was acid hydrolyzed and analyzed for the presence of monosaccharides by paper chromatography. The results are shown in table 10. In addition to the expected ribose, two other sugars designated X and Y were found. Monosaccharides X and Y were deciphered as sugar alcohols based on the following criteria. (1) Neither sugar reacted with indicator sprays M2 or M5 (see table legend) suggesting that they were neither reducing sugars nor non-reducing sugars of the methyl-pentose type. (2) Both sugars reacted with indicator spray M1 very quickly suggesting that they were sugar alcohols and this was confirmed by their reactions with indicators M3 and M4. (3) Sugar X was also found to be ninhydrin positive (indicator M6) suggesting that it probably contains an amino group. (4) Sugar X migrated with an R_f value similar to that of ribitol in the 3 solvent systems (table 10).

TABLE 10. Carbohydrate analysis of HS3 by paper chromatography

sugar	Rg^+ solvent VIII	IX	X	colour reaction with reagent M1	M2	M3	M4	M5	M6
X	2.25	1.7	3.0	+	-	+	+**	-	+
Y	1.3	1.15	1.1	+	-	+	+**	-	-
ribose	3.3	1.8	4.5	+	+	+	+*	+	-
ribitol	2.2	1.7	3.1	+	-	+	+**	-	-
mannitol	1.3	1.2	1.05	+	-	+	+**	-	-
sorbitol	1.3	1.2	1.5	+	-	+	+**	-	-

* indicates grey colour

** mauve colour appeared when respective sugars reacted with spray

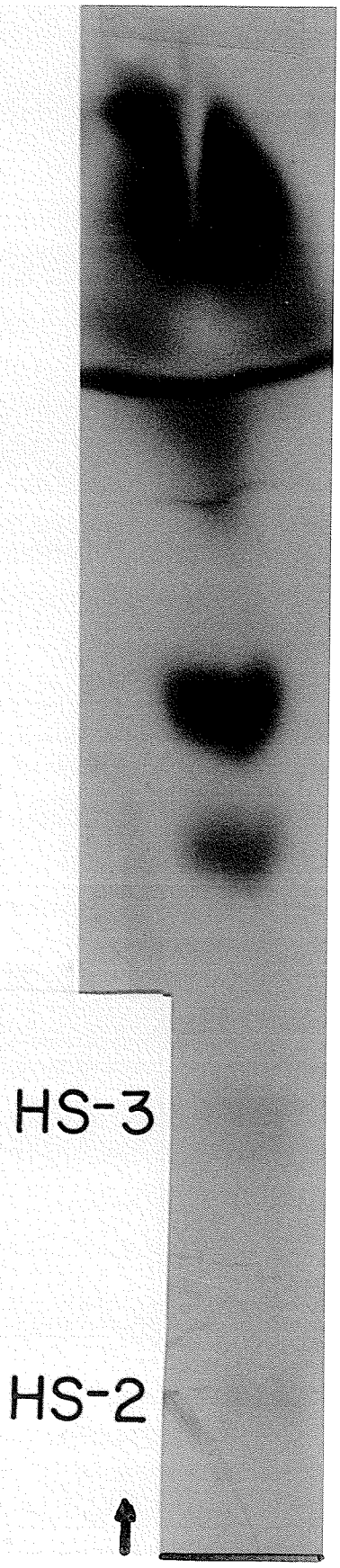
+ Rg indicates R_f values of sugars with respect to glucose as standard

M1: sodium periodate-permanganate, M2: p-anisidine, M3: bromocresol purple, M4: vanillin, M5: benzidene-TCA, M6: ninhydrin

(5) Sugar Y migrated with R_f values similar to those of mannitol and sorbitol in solvents VII and IX but was distinguished from sorbitol with solvent X.

These results suggested that sugar Y was mannitol. To test this possibility out further, we attempted to label HS3 with ^{14}C-mannitol. LéJohn et al. (1978) have shown that ^{14}C-labelled HS compounds can be isolated from the membrane area by cold osmotic shock treatment. Small cultures of Achlya (see Methods) were grown for 21 h, harvested and resuspended in starvation buffer supplemented with 50 µCi of ^{14}C-mannitol. These were incubated for 5 h then harvested and osmotically shocked. The osmotic shock fluid was then concentrated, desalted on Sephadex-G10 columns, dried in vacuo and residue dissolved in water before chromatographing on PEI-cellulose thin layer plates in solvent I. An autoradiogram of this is shown in figure 15. Both HS3 and HS2 contain ^{14}C-radioactive material. To confirm the presence of ^{14}C-mannitol HS3 was eluted from the PEI-cellulose with 2M TEAB buffer, the sample evaporated to dryness and washed several times with water. Unlabelled mannitol was added to the isolate, the sample acid hydrolyzed, and the hydrolysate was chromatographed on paper in solvent X. Mannitol was located and found to

Figure 15: Autoradiogram showing separation of ^{14}C-mannitol labelled HS compounds chromatographed on PEI-cellulose in solvent I. ^{14}C-labelled HS extracted from Achlya by the osmotic shock technique (see Methods).


HS-3
HS-2

contain ^{14}C-label. No estimation could be made as to the specific activity of the ^{14}C-mannitol in HS3 as quantities were limiting. These results suggest that sugar Y is mannitol. The same procedure was done for HS2 (also seen in Figure 15) which will be described in the section "Carbohydrate Analysis" of HS2 and 1.

The estimation of these two sugars has been carried out on various fragments from HS3 produced by chemical and enzymatic methods. The results which will be presented later, (see "Analysis of Fragments of HS3") indicate that there is one molecule of sugars X and Y per molecule of HS3.

5. Phosphate Analysis

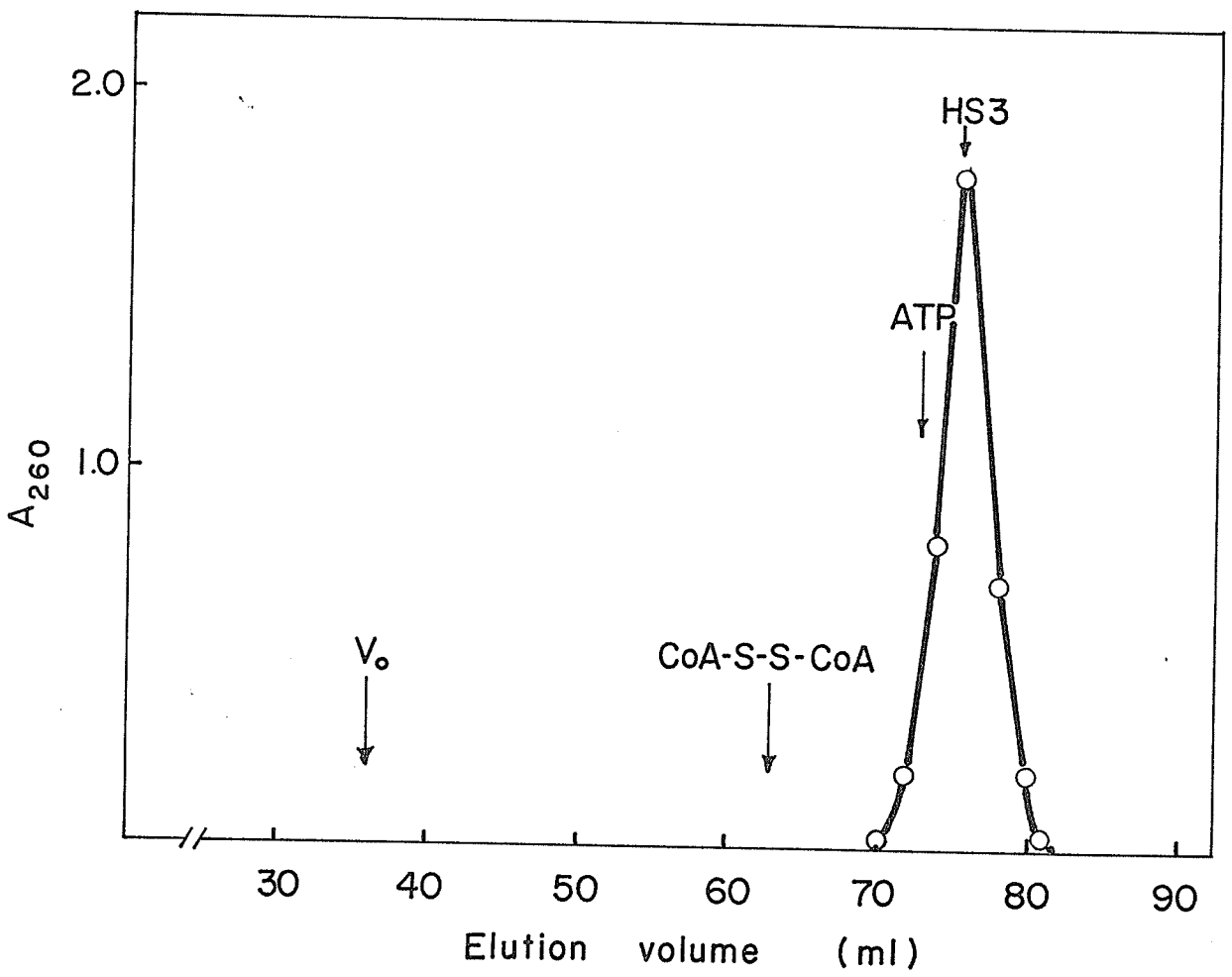
Total phosphate was determined after ashing by the method of Lowry et al. (1954) and correlated with ribose and nucleobase content. The results shown in table 7 indicate that HS3 has 8 phosphates per molecule.

6. G50 Chromatography

HS3 has been shown to contain adenosine, uridine, glutamate, mannitol, sugar X (amino polyol) and 8 phosphates. These results indicate that HS3 is a large complex molecule, having a minimum molecular weight of approximately 1,792 based on the sum of the molecular weights of the components (since uncertainty exists as to the exact nature of sugar X a molecular weight of 152 that of ribitol, was used).

Chromatography of HS3 on Sephadex-G50 (shown in figure 16) indicated that HS3 eluted from the column in 75ml; ATP, molecular weight 507, eluted in 73ml and the oxidized coenzyme A dimer, molecular weight 1535, eluted in 63ml. Based on molecular weight, HS3 might be expected to elute from the column very near the coenzyme A dimer rather than ATP. However, these results may suggest that HS3 is a very compact molecule smaller in size compared to the large coenzyme A dimer.

Figure 16: Chromatography of HS3 on Sephadex-G50. HS3 was applied to a (1 x 100cm) Sephadex-G50 column and eluted with 50mM TEAB buffer pH 8.0. Arrows indicate the elution volumes of ATP, Coenzyme A dimer (CoA-s-s-CoA) and the void volume (V_o).


2.0
1.0
A_{260}
HS3
ATP
V_o
CoA-S-S-CoA
30
40
50
60
70
80
90
Elution volume (ml)

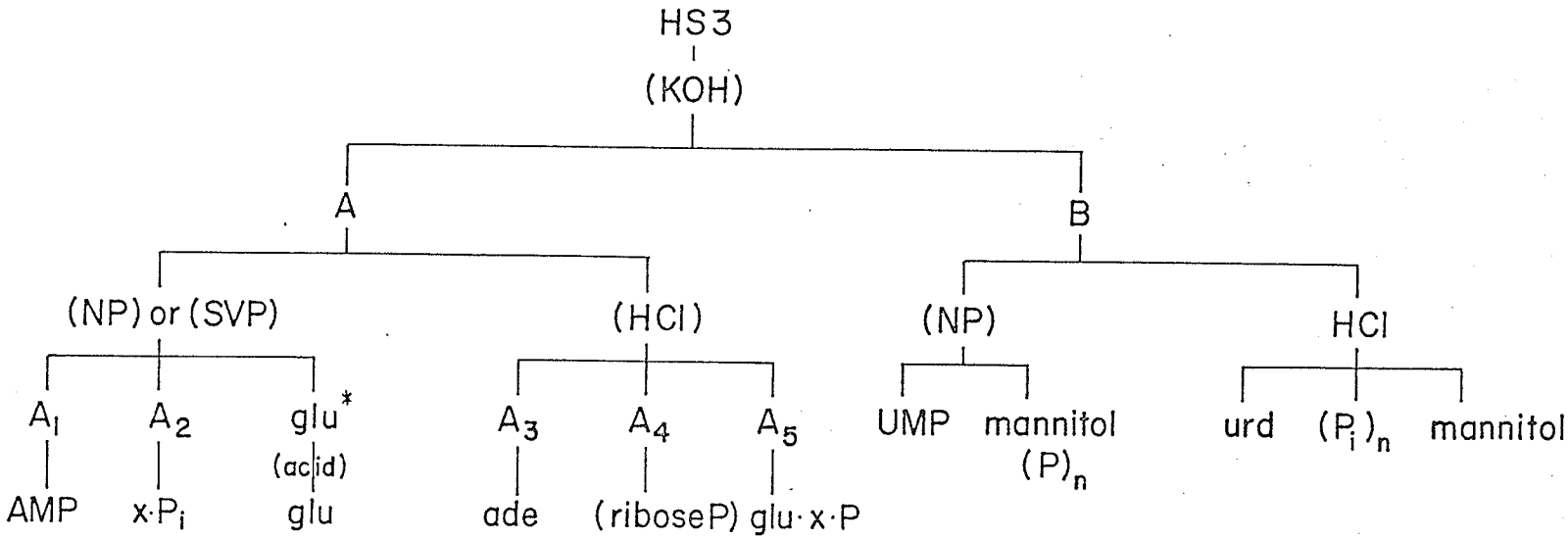
7. Alkali Hydrolysis of HS3 and Analysis of Fragments

HS3 was fragmented chemically and enzymatically. The fragments were isolated after column chromatography and analyzed for their components and possible structure. Figure 17 gives a summary of the treatments and the results which are discussed in the following sections.

^{32}P-labelled HS3 was incubated in 0.33N KOH for 20 h at 37^{o}, and the mixture fractionated on Sephadex-A25 (figure 18). Two uv-light absorbing products each containing ^{32}P-radioactivity were detected and are designated as fragments A and B. The fractions of both peaks were collected and desalted on Sephadex-G10 (as described for HS compounds) and analyzed as described below.

A study of the covalent bond that is sensitive to alkali treatment (causing HS3 to degrade to fragments A and B) led to the analysis of HS3 for a carboxylic phospho-anhydride bond (acylphosphate group). The method used is based on the fact that acyl-phosphates react rapidly with hydroxylamine (Lipmann and Tuttle 1945) forming hydroxamic acids, which in the presence of ferric salts produce red to violet complexes which are

Figure 17: Schematic diagrams of the chemical and enzymatic fragmentation studies of HS3. This diagram should be used as a reference (summary) for the following sections on Achlya HS3.
NP - nucleotide pyrophosphatasee
SVP- snake venom phosphodiesterase


HS3
(KOH)
A
B
(NP) or (SVP)
(HCl)
(NP)
HCl
A1
A2
glu*
(acid)
A3
A4
A5
UMP
mannitol
(P)n
urd
(Pi)n
mannitol
AMP
x·Pi
glu
ade
(riboseP)
glu·x·P

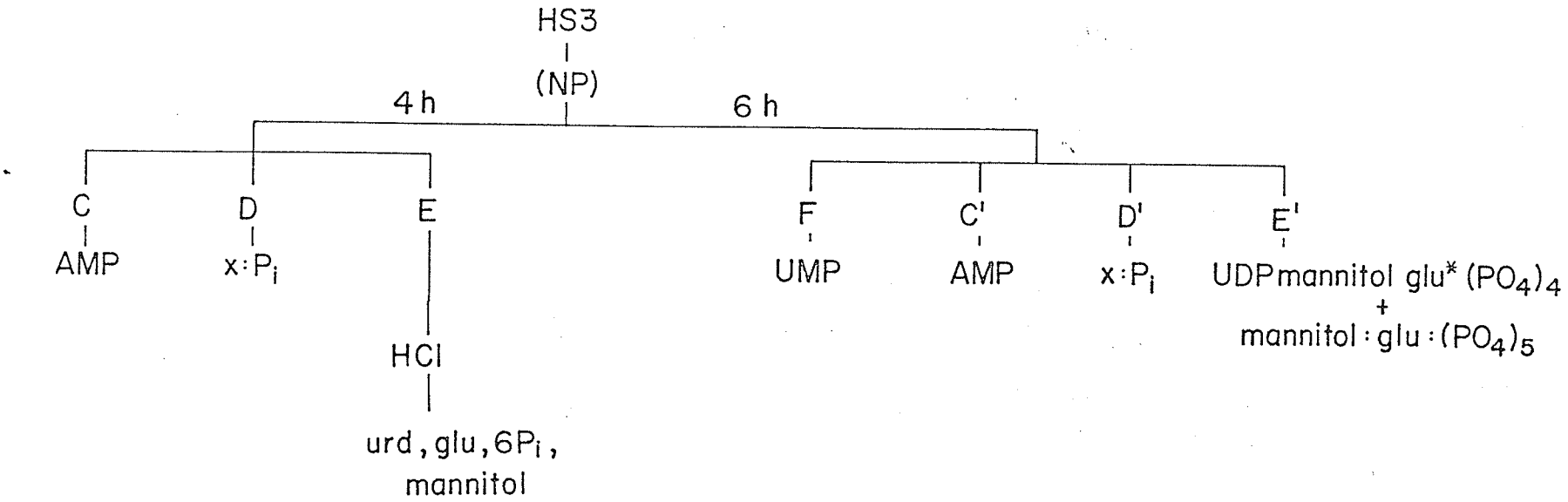
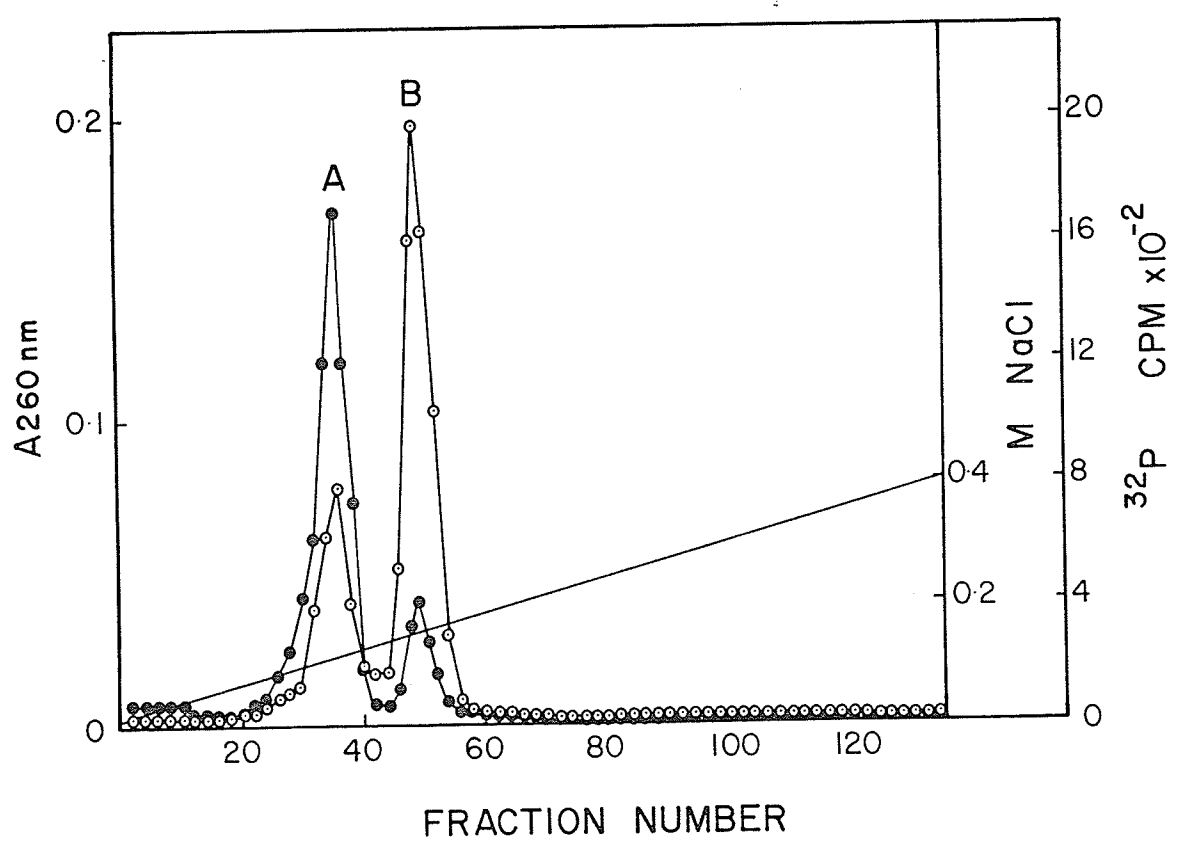

HS3
(NP)
4 h
6 h
C
D
E
F
C'
D'
E'
AMP
x:Pi
UMP
AMP
x:Pi
UDP
mannitol glu* (PO4)4
+
mannitol : glu : (PO4)5
HCl
urd, glu, 6Pi,
mannitol

Figure 18: Analysis of the products of KOH hydrolysis of HS3 on DEAE-Sephadex-A25 column. Ten A_{260} units of HS3 was incubated with 0.33N KOH at 37^{o} for 20 h. The hydrolysate was diluted 10-fold with 0.05M sodium formate and applied to a DEAE-Sephadex-A25 column (1x10cm) preequilibrated with 0.01M NaCl:0.05M sodium formate pH 3.6. The products were eluted using a 500ml (0.01-0.4M) gradient of NaCl in 0.05M sodium formate, pH 3.6. Four ml fractions were collected and analyzed for A_{260} (●) and ^{32}P (O) properties. Fractions under peaks A and B were pooled separately, desalted on Sephadex-G10 columns and resuspended in known volumes of distilled water.


A
B
0·2
0·1
0
A260nm
20
40
60
80
100
120
FRACTION NUMBER
0·4
0·2
M NaCl
20
16
12
8
4
0
32P CPM x10-2

quantitated colorimetrically. As shown in table 11, HS3 contained approximately one acyl-phosphate linkage per purine-bound ribose. Fragment A and B when tested showed negative results suggesting that fragments A and B might be linked by a carboxcylic acid-phosphate bond. Since this method also measures thiol-esters , HS3 was analyzed for the presence of thiol groups after alkline treatment, using the nitroprusside method described by Grumert and Phillips (1951). HS3 did not contain any thiol groups, indicating that the covalent linkage between fragments A and B probably involves a carboxyl-phosphate bond.

The properties of this type of covalent linkage have been described by Stadtman (1957) with respect to the compound acetyl phosphate. The carboxyl-phosphate bond in this compound is unstable in solution at low or high pH and at elevated temperatures. Neutral solutions of acetyl phosphate may be stored for months without appreciable decomposition.

Identical fragments as those produced by KOH hydrolysis of HS3 were obtained on rechromatography (at pH 3.6) of some preparations of HS3 which had

Table 11. Analysis of HS3 and fragments A and B of KOH hydrolysis for the presence of a carboxyphospho anhydride group

compound	nmoles	
	acyl-phosphate*	ribose
HS3	50	55
fragment A	-	80
fragment B	-	85**

*authentic acetyl phosphate used for standard curve

**ribose estimated after acid hydrolysis and sodium amalgam reduction (see table 14)

been stored for prolonged periods or repeatedly frozen. However, analysis of these preparations indicated that degradation of the molecule occurred upon rechromatography, the stock solutions showing intact molecules. This would suggest that this bond in HS3 is relatively unstable as is the case in acetyl phosphate. HS3 was found to be relatively stable for a few hours at temperatures up to 37^{o} at pH 7.

Fragment A (see figure 18)

An aliquot of fragment A material was chromatographed one-dimensionally on PEI-cellulose in modified solvent I (see Methods) and on cellulose paper in solvent III. The results are shown in table 12. Material from fragment A comigrated in both solvent systems with authentic ADP. The uv-light absorption scan of this material was similar to that of ADP. Figure 19 shows the absorption scan of fragment A at pH7. The spectral data for fragment A and authentic ADP is shown in table 13. Fragment A absorbs maximally between 257-259 over the pH range. The minimum remains constant

Table 12. Chromatographic analysis† of fragments produced by KOH hydrolysis of HS3

Fragment or standards*	R_f solvent I+	solvent III
A	0.57	0.27
B	0.21	0.85
*ADP	0.60	0.27
*GTP	0.22	0.55
*UTP	0.70	0.75
*P_i	0.76	0.99

+ solvent is modified solvent I

‡ chromatographed on paper in solvent III and on thin layer cellulose plates in modified solvent I.

Figure 19: Ultraviolet absorption spectrum of fragment A in 0.01M phosphate buffer pH 7.

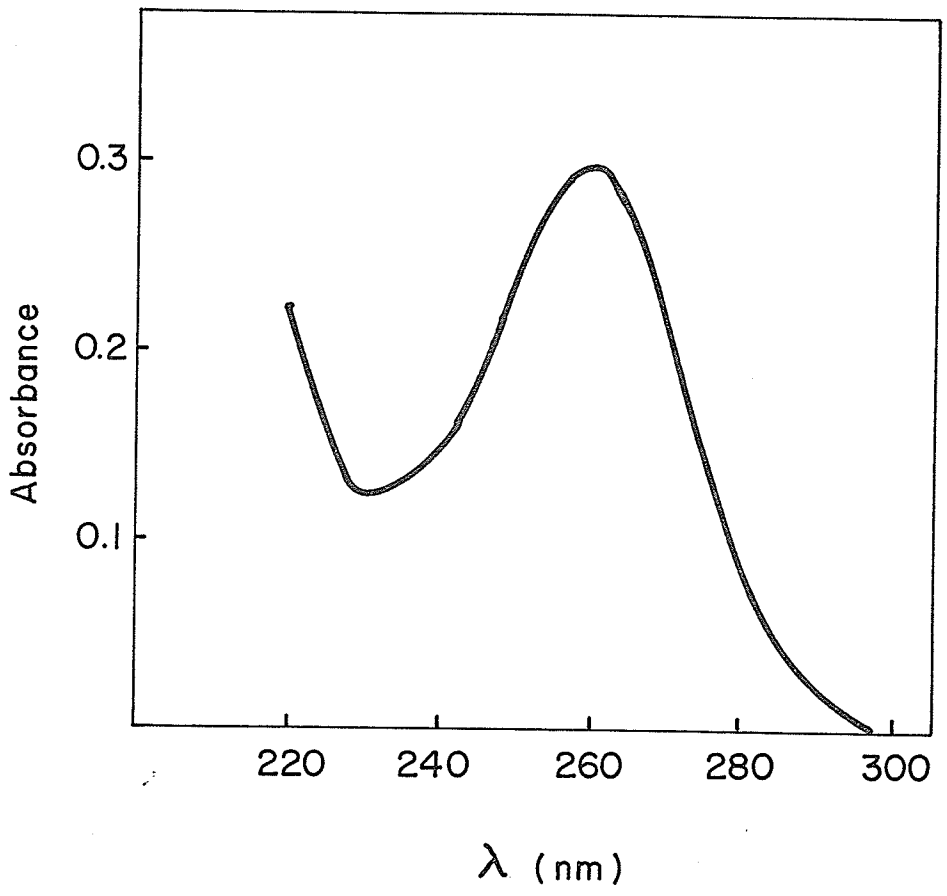

Absorbance
0.3
0.2
0.1
220
240
260
280
300
λ (nm)

Table 13. Spectral data for fragment A of KOH hydrolyzed HS3 and ADP.

compound	pH	λ nm max	λ nm min	Absorbance ratios 250/260	280/260	290/260
Fragment A	1	257	230	0.86	0.25	0.08
	7	259	230	0.82	0.25	0.08
	12	259	230	0.83	0.24	0.04
*ADP	2	257	230	0.85	0.21	-
	7-12	259	230	0.78	0.16	-

* data for ADP taken from Burton (1969).

at 230nm from pH 1-12. ADP shows similar characteristics. Fragment A, however, has slightly higher 280/260 ratios than ADP. The extinction coefficient at 260nm (H_2O) of fragment A was estimated to be 13.0×10^3 based on the amount of ribose per A_{260} nm unit. This value is lower than that reported for authentic ADP which has an extinction coefficient at 260nm of 14.5×10^3 (Burton 1969).

A sample of fragment A material was digested by bacterial alkaline phosphatase and nucleotide pyrophosphatase and the products chromatographed two dimensionally on thin layer cellulose sheets. The uv-light absorbing product recovered comigrated (R_f 0.64 in the first dimension and 0.30 in the second dimension) with product A (see table 6 for clarification) and authentic adenosine.

Fragment A material was analyzed for the presence of glutamate (after acid hydrolysis) by two dimensional chromatography (as described for HS3). Fragment A contained glutamate that could only be recovered after acid or harsh KOH hydrolysis (0.5N at 100°for 2h)

A quantitative analysis of the adenine, ribose and phosphate in fragment A gave the results shown

in table 14. Fragment A contained 1 mole of ribose and 2 moles of phosphate per mole of adenine.

Fragment A was analyzed for carbohydrate (as described for HS3) and found to contain the polyhydric sugar X as well as the expected ribose. The carbohydrate content of fragment A was estimated by two methods and compared to the amount of ribose estimated by the orcinol procedure. Fragment A was acid hydrolyzed and treated with sodium metaperiodate as described by Dixon and Lipkin (1954). The periodate consumed was measured by the decrease in absorbance at 225nm (with respect to the controls) and compared with a ribose standard to quantitate the amount of carbohydrate. The results given in table 15 show that while the orcinol method gave results of 52 nmoles of ribose in fragment A, periodate oxidation determined that there was 100 nmoles of carbohydrate. These results indicate that sugar X consumes an identical amount of periodate per molecule as ribose suggesting that sugar X is at least a five carbon sugar, and that fragment A contains 1 mole sugar X per mole ribose. The second method used to quantitate sugar X was the determination of formaldehyde released after periodate oxidation

Table 14. Quantitative analysis of fragments A and B of KOH hydrolyzed HS3

Fragment	nmoles			ratios		
	ribose	base	PO_4	ribose/PO_4	ribose/base	base/PO_4
A	8.5	8.0*	16	1:1.9	1:1.1	1:2
B	8.0**	8.0*	48	1:6	1:1	1.6

*. Extinction coefficient of 13.3×10^3 and 9.9×10^3 were used to calculate the nmoles of base in acid hydrolyzed samples of fragments A and B respectively

**Ribose analyzed by orcinol after 1 hour acid hydrolysis and 12 h pretreatment with sodium amalgam (1%).

Table 15. Estimation of carbohydrate content of fragment A

Method	nmoles
orcinol* (ribose)	52*
+periodate consumption	100**
+formaldehyde determination	115**

* measures purine bound ribose

** based on authentic ribose as standard

+Fragment A was acid hydrolyzed for 20 h in 1N HCl at 105° *in vacuo*, the acid was removed by evapomix and then carbohydrate estimated by by the periodate method, or formaldehyde determination after periodate oxidation.

of the sugars. Formaldehyde is released from the terminal CH_2OH groups by periodate oxidation. Ribose, on periodate oxidation, releases 1 mole formaldehyde per mole ribose. The colorimetric method used to determine formaldehyde was based on the procedure of Lambert and Neish (1950). The amount of formaldehyde released from the treated sample of fragment A was estimated as 115 nmoles (table 15). Since the sample contained 52 nmoles of ribose, which would release 52 nmoles formaldehyde, sugar X then released 63 nmoles of formaldehyde. Assuming sugar X to contain one terminal CH_2 OH (sample had been acid hydrolyzed for 20 h *in vacuo* to release PO_4) we would expect approximately 52 nmoles of formaldehyde from ribose and 52 nmoles from sugar X for a total of 104. The value of 115 nmoles obtained is close to the theoretical.

These results indicate that there is 1 mole of sugar X per mole of ribose in fragment A; that sugar X is a five carbon sugar and that it has at least one terminal CH_2OH group, indicating its polyhydric nature as previously discussed.

A sample of fragment A was digested by nucleotide pyrophosphatase alone (see legend figure 20 for details) and the products separated on Sephadex-A25 (figure 20) into two components designated subfragments A1 and A2. Subfragment A1 absorbed uv-light and contained ^{32}P-radioactivity. Subfragment A2 did not absorb uv-light but did contain ^{32}P-radioactivity.

Analysis of subfragment A1 gave the following results. (1) It comigrated with authentic AMP in two solvent systems (table 16). (2) Fragment A1 was susceptible to periodate oxidation which resulted in a change in the R_f value of the treated sample (table 17). (3) Authentic 5'AMP gave identical results as fragment A1, whereas 2' or 3'AMP showed no change in the R_f values after periodate treatment. 2' or 3'AMP is not susceptible to periodate because of the blocking phosphate group at the 2' or 3' position. (4) Amino acid analysis (as described in Methods) of fragment A1 was negative indicating that glutamate was not present. These results suggest that fragment A1 is 5'AMP.

Figure 20: Nucleotide pyrophosphatase digestion of fragment A. Approximately 10 $A_{260\ nm}$ units of fragment A was treated with nucleotide pyrophosphatase for 2 hours at 37^{o} in 0.2M tris-HCl, pH 7.5 and 0.02M $MgCl_2$. Enzyme was used at 1 $\mu g/A_{260}$ unit. The mixture was diluted to 2 ml with 50 mM formate buffer, pH 3.6 and loaded on a Sephadex-A25 column (1x10cm) preequilibrated with 0.01M NaCl:50mM sodium formate buffer,pH 3.6. Products were eluted with a 200 ml linear (0.01-0.2M) NaCl gradient in 50mM formate buffer, pH 3.6. Fractions (2ml) were collected and analyzed for A_{260}(●) and ^{32}P radioactivity (O). Fractions under peaks labelled A1 and A2 were collected and desalted on Sephadex G10 columns. Sub-fragments were resuspended in known volumes of water.

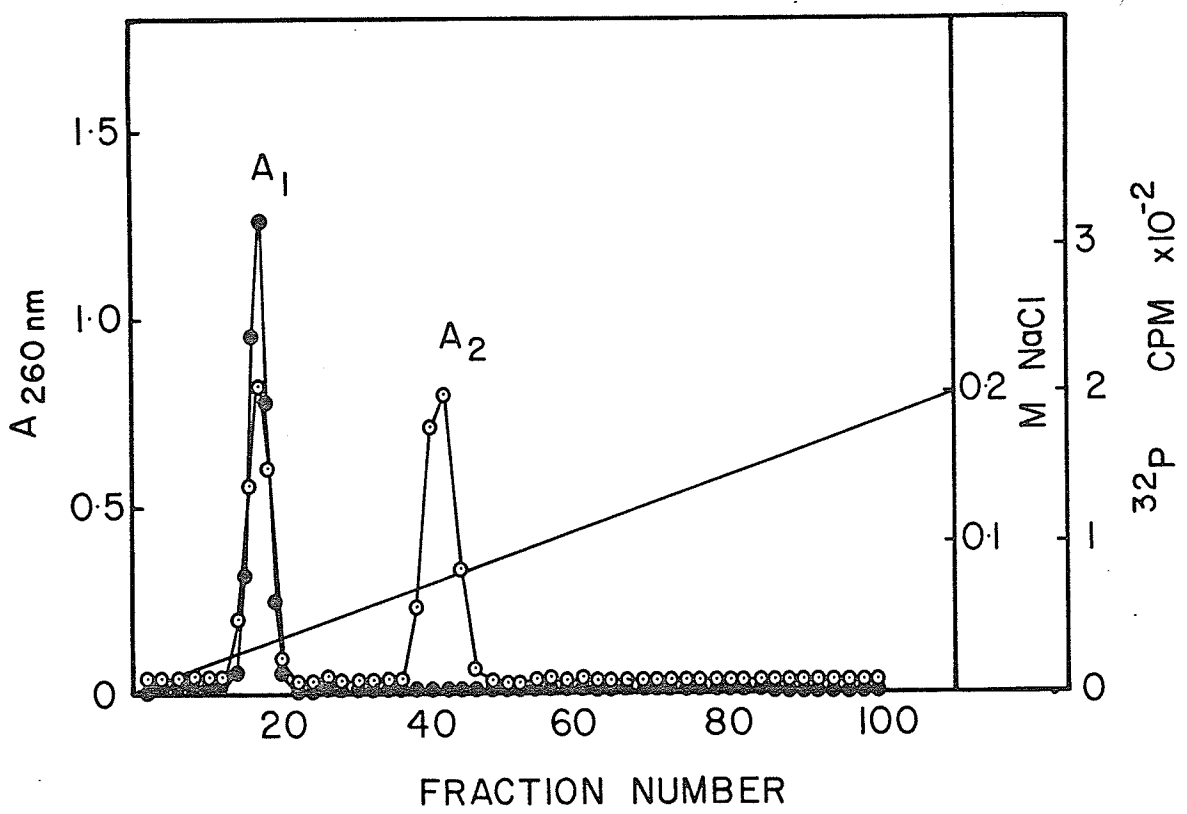

1·5
1·0
0·5
0
A 260 nm
A1
A2
0·2
0·1
M NaCl
3
2
1
0
32P CPM x10-2
20
40
60
80
100
FRACTION NUMBER

Table 16. Analysis of subfragments A1 and A2 produced by nucleotide pyrophosphatase digestion of fragment A.

A. Chromatography† of subfragments and standards

Fragment	Rf*	
	modified solvent I	solvent III
A1	0.69	0.20
A2	0.76	0.99
5'AMP	0.69	0.20
^{32}Pi	0.76	0.99
2'and 3'AMP	0.69	0.14, 0.18

*located by ^{32}P-radioactivity and (or) uv-light absorbance

† on paper in III, on cellulose in solvent I

B. Carbohydrate Estimation

Fragment	nmoles ribose (orcinol)	nmoles formaldehyde determination*	nmoles amino sugar determination+
A1	13.5	15	-
A2	0.75	14(28)	14

* samples acid hydrolyzed in IN HCl in vacuo at 105°. Values outside bracket obtained from 1 h acid hydrolyzed sample; value inside bracket from 20 h acid hydrolyzed sample.

Table 17. Periodate oxidation * of fragment A1.

Fragment	periodate treatment	R_f
A1	-	0.60
A1	+	0.53
controls		
5'AMP	-	0.60
5'AMP	+	0.53
2' or 3' AMP	-	0.60
2' or 3'AMP	+	0.60
adenine	-	0.53

* The periodate oxidation method of Cashel and Kalbacher (1969) was used

+ components or products observed under uv-light when chromatographed on thin layer cellulose sheets in modified solvent I

-, untreated; +, treated

Subfragment A2 contained ^{32}P-radioactivity equivalent in amount to that in A1 (figure 20). It comigrated with P_i in both solvent systems (table 16). Carbohydrate analysis (as described in Methods) showed it to contain sugar X which was dissociated from the phosphate by acid hydrolysis suggesting a covalent linkage. It should be pointed out that after 20 h, acid hydrolysis of subfragment A2 in 1N HCl (used for carbohydrate analysis) sugar X could not be located by the ninhydrin reagent suggesting that the amino function had been hydrolyzed. Glutamate analysis of subfragment A2 gave negative results suggesting that neither A1 nor A2 contained glutamate before or after acid hydrolysis. The unfractionated products of nucleotide pyrophosphatase hydrolysis, however, contained glutamate that was identified chromatographically after acid (1N HCl for 1 h *in vacuo* at 105°) or alkali (0.5N KOH for 1 h at 105°). Thus it would appear that glutamate was present in HS3 in a modified form (designated glutamate*) which was released by nucleotide pyrophosphatase, but converted to glutamate (as analyzed by thin layer chromatography) after acid or KOH hydrolysis.

Carbohydrate estimation of subfragments A1 and A2 (table 16) shows that subfragment A1 contained purine-bound ribose (13.5 nmoles) as estimated by the orcinol procedure. An equivalent amount of ribose was quantitated in subfragment A1 by the colorimetric formaldehyde determination method after the acid hydrolyzed sample had been oxidized by periodate (table 16). Subfragment A2 did not react with orcinol; however, a sample acid hydrolyzed for 1 h released 14 nmoles of formaldehyde after periodate oxidation but after acid hydrolysis for 20 h released 28 nmoles. Formaldehyde was not released in the untreated sample (non-acid hydrolyzed). These values are based on a ribose standard which releases 1 mole formaldehyde per mole ribose. These results indicate that after 1 h hydrolysis one terminal CH_2OH group is exposed which on oxidation by periodate is released as formaldehyde. On this basis there is one mole sugar X per mole ribose in fragment A. However, 20 h hydrolysis of A2 results in 2 terminal CH_2OH groups and double the formaldehyde released by periodate. This suggests that the phosphate on sugar X is attached to a terminal CH_2OH group

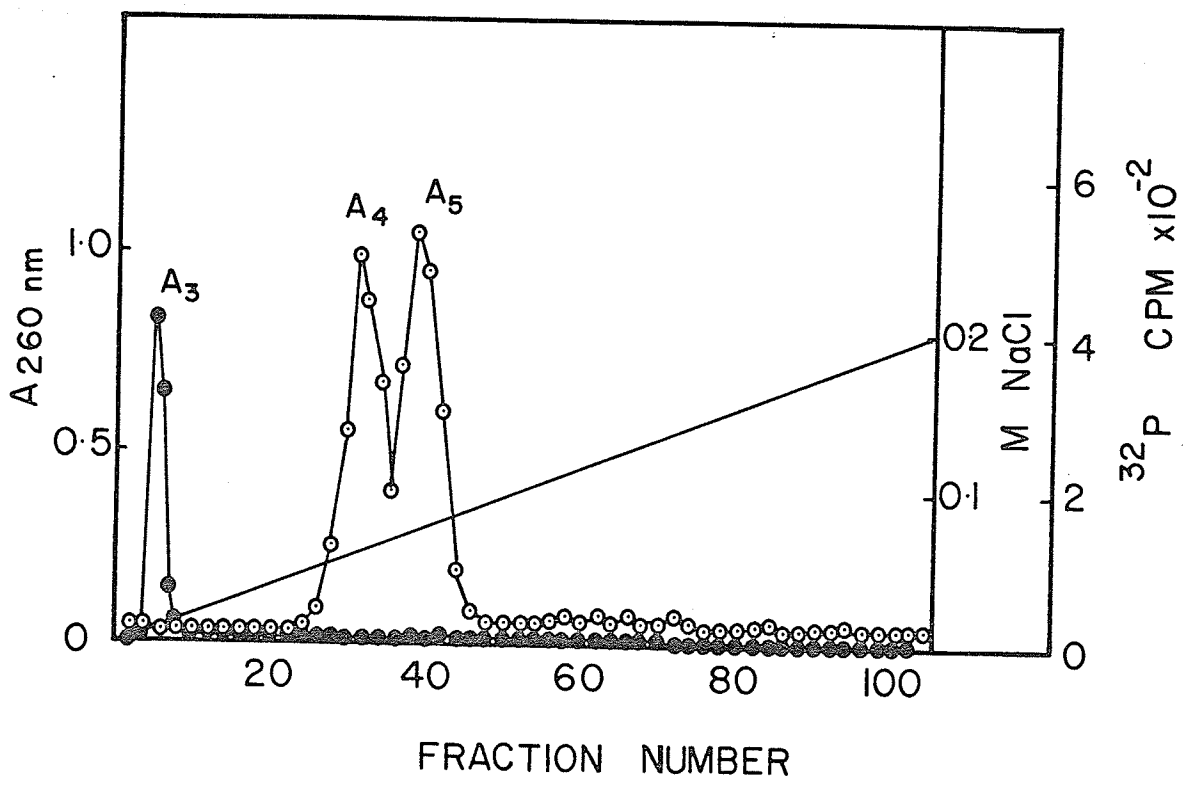
and is released after 1h hydrolysis. Hydrolysis of subfragment A2 for 20 h released another component which may be the amino function allowing the measurement of another terminal CH_2OH group.

Colorimeteric determination of the amino group of sugar X on subfragment A2 by the method of Moore and Stein (1954) indicated that there was one mole of the amino sugar X per mole of ribose in fragment A1 (table 16). Thus sugar X appears to be a five carbon polyhydric sugar with an amino substituent. Since it comigrates with ribitol, it might be an amino derivative of ribitol but this is purely speculative.

Nucleotide pyrophosphatase therefore, cleaved fragment A into AMP, $^{'}X^{'}MP$ and glutamate*. Similar products were obtained with snake venom phosphodiesterase digestion of fragment A. Therefore, fragment A appears to be $5^{'}ADP\text{-}^{'}X^{'}$ with glutamate* attached.

A sample of fragment A was subjected to limited acid hydrolysis (see figure 21 legend) and the products fractionated on Sephadex-A25 (figure 21) into three new subfragments called A3, A4 and A5. Subfragment A3 absorbed uv-light but did not contain ^{32}P label. It was identified chromatographically

Figure 21: Partial acid hydrolysis of fragment A. Approximately 5 A_{260} nm units of fragment A was hydrolyzed *in vacuo* in 1N HCl at 105° for 1 h. Acid was removed by evaporation, the residue dissolved in 50mM formate pH 3.6 buffer and loaded on a Sephadex-A25 column (1 x 10cm) preequilibrated with 0.01M NaCl in 50mM formate buffer pH 3.6. Products were eluted with a 200ml linear (0.01-0.2M) NaCl gradient in 50mM formate pH 3.6 buffer. Fractions (2ml) were collected and analyzed for A_{260} (●) and ^{32}P-radioactivity (O). Fractions under peaks A3, A4 and A5 were collected, desalted and resuspended in equivalent volumes of distilled water.


A 260 nm
1·0
0·5
0
A3
A4
A5
20
40
60
80
100
FRACTION NUMBER
0·2
0·1
M NaCl
6
4
2
0
32P CPM x10-2

as adenine (R_f 0.12 in solvent III). Subfragments A4 and A5 had equal amounts of ^{32}P-radioactivity. Carbohydrate analysis by paper chromatography showed that A4 contained ribose which was estimated by the orcinol procedure. Subfragment A5 contained the amino sugar X. Ribose contamination in A5 from A4 was negligible. Glutamate (as analyzed by acid hydrolysis and 2 dimensional chromatography) was found to be associated with subfragment A5.

As subfragment A5 contained only sugar X and phosphate, glutamate* must be linked to it either through the phosphate or sugar X itself. The possible covalent linkage of glutamate* will be discussed fully (see Discussion). Thus acid hydrolysis degrades fragment A into adenine, ribose phosphate and sugar X-phosphate (glutamate). The arrangement of the components in fragment A would then be either glutamate*-XppA or Xp (glutamate*) pA.

Fragment B Analysis (see figure 18)

Fragment B was analyzed by similar methods as described for fragment A. Fragment B migrated with an R_f similar to GTP in modified solvent I, but ahead of UTP in solvent III (table 14). These results suggest that it might be a highly phosphorylated uridine nucleotide. The uv-light absorption spectrum of this material at pH 7.0 is shown in figure 22. The spectral data of fragment B is given in table 18. Fragment B absorbs maximally at 263nm over the pH range, and shows a shift in the minimum from 232 to 245 at alkaline pH. This is characteristic of uridine and its related nucleotides. Fragment B has higher $A_{280/260}$ and $A_{290/260}$ ratios compared with uridine. A shift in the minimum is evident in HS3, from 232 to 235nm whereas the shift in minimum of fragment B is from 232 to 245nm.

As can be seen in figure 18, the absorbance at 260nm of fragments A and B were disproportionate, however, the absorbance of fragment B increased by 78% after desalting in Sephadex G10. This result shows that the lowering of the extinction

Figure 22: Ultraviolet absorption spectrum of fragment B at pH 7.0 PO_4, pH 7.0.

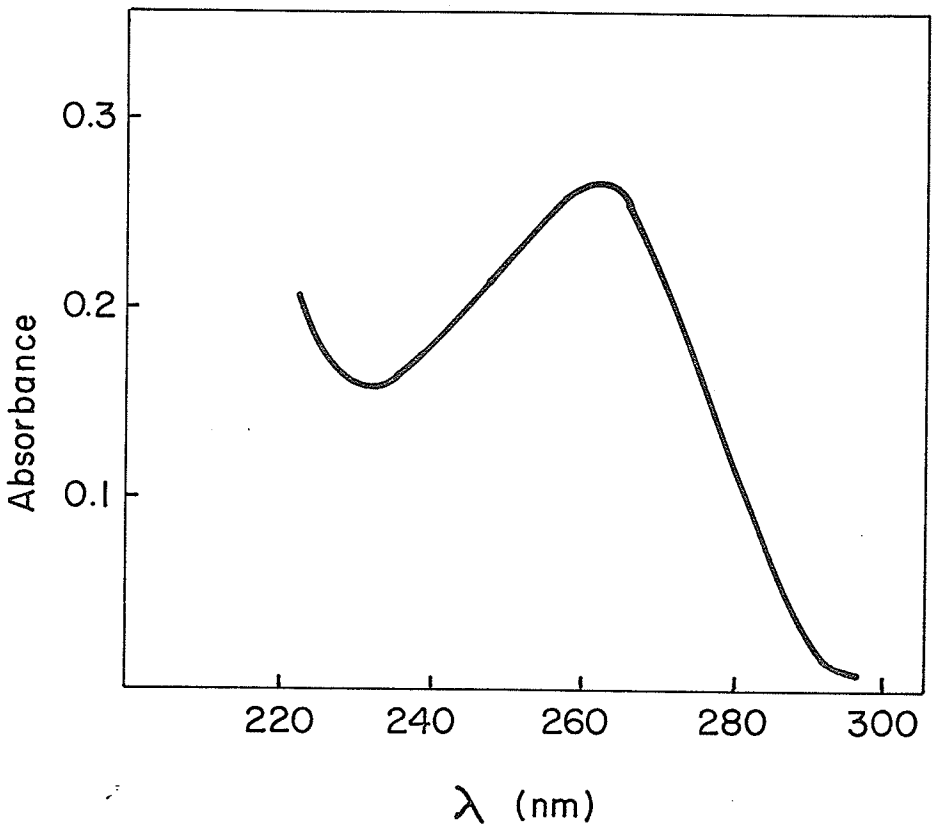

0.3
0.2
0.1
Absorbance
220
240
260
280
300
λ (nm)

Table 18. Spectral data of fragment B from KOH hydrolysis of HS3

compound	pH	λ nm max	λ nm min	RATIOS $A_{250/260}$	RATIOS $A_{280/260}$	RATIOS $A_{290/260}$
Fragment B	7	263	232	0.85	0.55	0.20
	12	263	245	0.86	0.56	0.22
uridine*	7	262	231	0.74	0.35	0.03
	12	262	242	0.83	0.29	0.02

*spectral ratios for uridine taken from Burton (1969).

coefficient in HS3 (discussed earlier) is mainly due to the salt effects on the absorption of the uracil entity, however, a 20% decrease in the extinction coefficient of fragment A was observed in salt solutions. Hydrolysis of fragment B by 1N HCl for 1 h at 105^{o} followed by removal of the acid produced maximum absorption. Using the extinction coefficient at 260nm of 9.9×10^{3} for fragment B (acid hydrolyzed) a mole ratio of 1:1 was calculated for the nucleobases in fragments A and B (table 14).

After digestion of fragment B by nucleotide pyrophosphatase and alkaline phosphatase, the products of hydrolysis were chromatographed two dimensionally on thin layer cellulose plates. A single uv-light absorbing material that comigrated with uridine and product B was observed (see table 6 for clarification) having an R_f of 0.38 in the first dimension and 0.76 in the second dimension. Fragment B did not contain glutamate.

Fragment B did not react with orcinol directly, and after reduction with sodium amalgam for 20 h showed only 30-35% reactivity of the expected

amount of pyrimidine-bound ribose. When fragment B was acid hydrolyzed for 1 h with 1N HCl *in vacuo* at 105°, then treated with sodium amalgam, there was 100% recovery of pyrimidine-bound ribose (table 16) giving a mole ratio of 1:1 between the purine-bound ribose in fragment A and pyrimidine-bound ribose in fragment B.

The reduction of pyrimidine nucleotides is required prior to analysis by orcinol (Haavaldsen et al. 1958). The hydrogenation of common pyrimidine nucleotides only requires 4 h at room temperature by sodium amalgam, to destabilize the sugar nitrogen base linkage. However, with fragment B, only 35% of the expected ribose was estimable by orcinol after 20 h of reduction suggesting that the reduction process is inhibited in fragment B. Kochetkov et al. (1964; 1971) have reported that hydrogenation of uridine dinucleotide sugar molecules is considerably retarded, compared to common uridine nucleotides. They have suggested that this effect may be the result of an intramolecular interaction (secondary structure) between the heterocyclic base and sugar residue in the pyrimidine diphosphate carbohydrate molecule.

Quantitation of the phosphates on fragment B resulted in a base to phosphate (or ribose to phosphate) ratio of 1:6 (table 14). The ^{32}P ratio between fragments A and B was found to be 1:3. Comparison of the organically bound phosphate per sample of fragment A and B confirmed the ratio of 1:3. Fragment A, therefore, contains 2 phosphates and fragment B, 6 phosphates.

Fragment B was analyzed for carbohydrate and found to contain sugar Y (table 10) which has been identified as mannitol (see "Carbohydrate Analysis of HS3"). Quantitative analysis of the carbohydrate in fragment B gave the results shown in table 19. A sample of fragment B was estimated for ribose by the orcinol procedure (after acid hydrolysis and sodium amalgam treatment) and was found to contain 70 nmoles. Estimation of the formaldehyde released, on oxidation of the sugars in fragment B by periodate, using ribose as standard (1 mole formaldehyde released per mole ribose) gave 3 times the amount of formaldehyde as ribose. This is consistent with a molecular ratio of 1:1 between mannitol and ribose in fragment B, since mannitol

Table 19. Carbohydrate estimation+ of fragment B from KOH hydrolysis of HS3

estimation by	nmoles
orcinol*	70
**formaldehyde determination	210
**periodate consumed	150

* ribose estimated by orcinol after 1 h acid hydrolysis in 1N HCl at 105° in vacuo, plus 12 h reduction with 1% sodium amalgam.

** sample hydrolyzed for 20 h, 1N HCl at 105° in vacuo, before determining the amount of formaldehyde released by periodate oxidation, or periodate consumed

\+ ribose used as standard

contains two terminal CH_2OH groups, whereas ribose contains one terminal CH_2OH group. Therefore mannitol, on periodate oxidation, would release 2 moles formaldehyde per mole formaldehyde released by ribose (70 nmoles), for a total of 210 nmoles. Measurement of the periodate consumed (based on a ribose standard) estimated the carbohydrate content in fragment B to be 150 nmoles. This is consistent with one mole of mannitol per mole ribose in fragment B as mannitol consumes 5 moles periodate per mole sugar and ribose consumes 4 moles periodate per mole. Since ribose was used as standard, the theoretical value would be (9/4 x 70 nmoles ribose) 157 nmoles. The estimated value (150 nmoles) is very close to the expected value. These data indicate that fragment B contains 1 mole of mannitol per mole pyrimidine-bound ribose.

Fragment B was completely dephosphorylated in 2 h at 37° using a mixture of bacterial alkaline phosphatase and nucleotide pyrophosphatase yielding uridine. Therefore, the effects of each enzyme on fragment B was studied. Bacterial alkaline

phosphatase was inactive on fragment B. Nucleotide pyrophosphatase hydrolyzed fragment B very slowly. After 4 h incubation, one product which absorbed uv-light was recovered which cochromatographed with UMP in two solvent systems (table 20). The second product contained ^{32}P-radioactivity (no uv-light absorption) and cochromatographed with $^{32}P_i$ on paper in solvent III, but with fragment B on PEI-cellulose in modified solvent I. The UMP product contained 12% of the radioactivity of the total ^{32}P-radioactivity which corresponds to 0.75 (or about 1.0) phosphate. Fragment B was insensitive to digestion by bovine spleen phosphodiesterase, which requires a free 5' hydroxyl group on the ribose for activity. Fragment B was insensitive to periodate oxidation as measured by a change in R_f when chromatographed on PEI-cellulose in modified solvent I (Cashel and Kalabacher 1969). However, fragment B was shown to consume a small amount of periodate measured spectrophotometrically as a loss in absorbance at A_{225}nm (Dixon and Lipkin 1954).

Therefore, fragment B contains uridine, mannitol

Table 20. Chromatographic analysis of the products produced by nucleotide pyrophosphatase digestion of fragment B

compound	Rf modified solvent I	Rf solvent III
Fragment B	0.38	0.86
product 1	0.76	0.71
product 2	0.39	0.93
UMP*	0.76	0.71
UTP*	0.74	0.82
P_i*	0.78	0.95

*standards

and 6 phosphates. A UDP structure is suggested by the nucleotide pyrophosphatase results. The inability of bovine spleen phosphodiesterase to cleave fragment B suggests that the phosphates are oriented at the 5' hydroxyl position on the nucleoside residue. This is also suggested by the periodate oxidation results. The inability of fragment B to be reduced with sodium amalgam might suggest a UDP-mannitol structure with 4 phosphates attached.

8. Nucleotide Pyrophosphatase Hydrolysis of HS3

^{32}P-labelled HS3 was hydrolyzed with nucleotide pyrophosphatase and the products separated on Sephadex-A25 (figure 23). Three identifiable products designated C, D and E were recovered. Only C and E contained uv-light absorbing materials whereas ^{32}P-radioactivity was present in all three.

Fragment C comigrated with authentic AMP in both solvent systems (table 21). Nucleoside analysis after enzymatic digestion of fragment C with bacterial alkaline phosphatase indicated that it

Figure 23: Chromatographic analysis of products of nucleotide pyrophosphatase digestion of HS3. Ten A_{260} units of HS3 was incubated with 0.1M tris-HCl pH 7.5, 0.02M $MgCl_2$, 1mg/ml enzyme for 4 h at 37°. The mixture was diluted with 0.05M sodium formate pH 3.6 buffer, loaded on Sephadex-A25 column (1 x 10cm) and eluted with a linear (0.1-0.4M) NaCl gradient in 50mM sodium formate pH 3.6. Fractions (4ml) were collected and analyzed for A_{260} nm (●) and ^{32}P-radioactivity (O). Fractions under peaks C, D and E were collected and desalted before analysis.

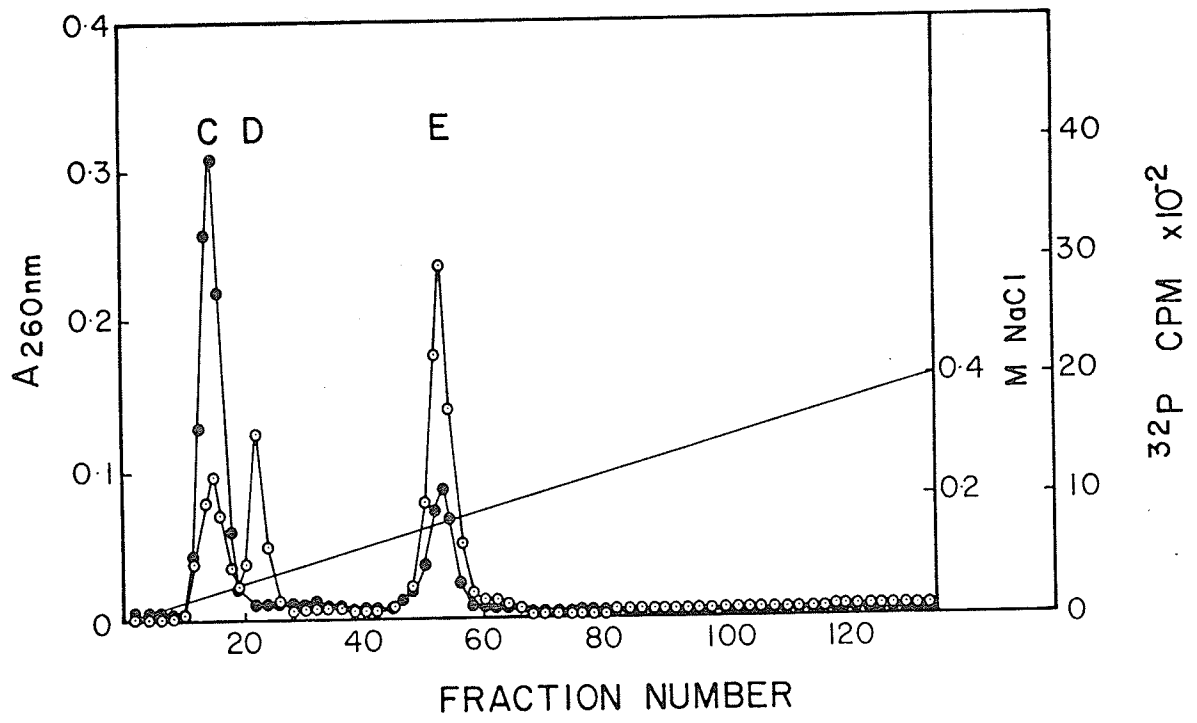

0·4
0·3
0·2
0·1
0
C
D
E
A 260nm
20
40
60
80
100
120
FRACTION NUMBER
0·4
0·2
M NaCl
40
30
20
10
0
32P CPM x10-2

Table 21. Chromatographic analysis† of fragments produced by nucleotide pyrophosphatase digestion of HS3

Product	R_f solvent I+	R_f solvent III
C	0.68	0.20
D	0.78	0.98
E	0.21	0.85
GTP*	0.22	0.55
UTP*	0.70	0.75
P_i*	0.76	0.98
5'AMP*	0.69	0.20
2'-3'AMP*	0.69	0.14-0.18

+ modified solvent I

* standards

† chromatographed on paper in solvent III and on thin layer cellulose in solvent I

contained adenosine. Fragment C did not contain glutamate either before or after acid hydrolysis when analyzed by two-dimensional chromatography. The ribose:phosphate:base ratio was computed as 1:1:1 (table 22). Fragment C material was susceptible to periodate oxidation (as described for subfragment A1 in table 17) which suggests a 5' phosphate linkage.

Fragment D migrated with P_i in both chromatographic systems (table 21). Both ^{32}P-radioactivity and colorimetric analysis for phosphate indicated that there were equivalent amounts of phosphate in fragments C and D (table 21). Fragment D did not contain glutamate (before or after acid hydrolysis). Carbohydrate analysis indicated fragment D to be a sugar (X) monophosphate.

Fragment E migrated slightly ahead of UTP during chromatography on paper in solvent III. On thin layer PEI-cellulose plates, fragment E migrated close to GTP in modified solvent I. The uv-light absorption between fragments C and E is not proportional; however a sample of fragment E (after acid hydrolysis) was found to contain

Table 22. Chemical analysis of fragments produced by nucleotide pyrophosphatase digestion of HS3

Fragment	nmoles ribose	nmoles base*	nmoles PO_4	ratios ribose/PO_4	ratios ribose:base	ratios base:PO_4
C	15	15	14	1:0.94	1:1	1:0.94
D	-	-	15	-	-	-
E	15**	15	94	1:6.2	1:1	1:6.2

* nmoles of base was estimated using an extinction coefficient of 14.5 x 10^3 for fragment C and 9.9 x 10^3 for an acid hydrolyzed sample of fragment E.

** ribose estimated in fragment E by orcinol after 1 h acid hydrolysis and 12 h sodium amalgam reduction.

an equivalent amount of base as fragment C (table 22). Fragment E was found to contain glutamate (after acid hydrolysis and two-dimensional chromatography). Fragment E was similar to fragment B which was produced by KOH hydrolysis except that fragment E contained glutamate.

Digestion of HS3 for 6 h with nucleotide pyrophosphatase resulted in the release of four products (figure 24) labelled C', D', E' and F. Analysis of C' and D' showed them to be identical to fragments C and D respectively in figure 23. The appearance of fragment F was coincident with the loss of uv-absorbing material in fragment E', suggesting that fragment F was released from fragment E' by enzymic action.

Fragment F was analyzed chromatographically and found to comigrate with 5'UMP in two solvent systems (table 23). Fragment F was found to contain a phosphate to base ratio of 1:1 (table 24). Fragment F did not contain glutamate before or after acid hydrolysis. These results suggest fragment F to be UMP.

Fragment E' migrated similar to fragment E

Figure 24: Chromatographic analysis of products of nucleotide pyrophosphatase digestion of HS3. Ten A_{260} nm units of HS3 was incubated with 0.1M tris-HCl pH 7.5, 0.02M $MgCl_2$, 1mg/ml enzyme for 6 h at 37°. The mixture was diluted with 0.05M sodium formate pH 3.6 buffer, loaded on a Sephadex-A25 column (1 x 10cm) and eluted with a linear (0.1-0.4M) NaCl gradient in 50mM sodium formate, pH 3.6. Fractions (4ml) were collected and analyzed for A_{260} nm (•----•) and ^{32}P-radioactivity (•——•). Fractions under were collected and desalted before analysis.

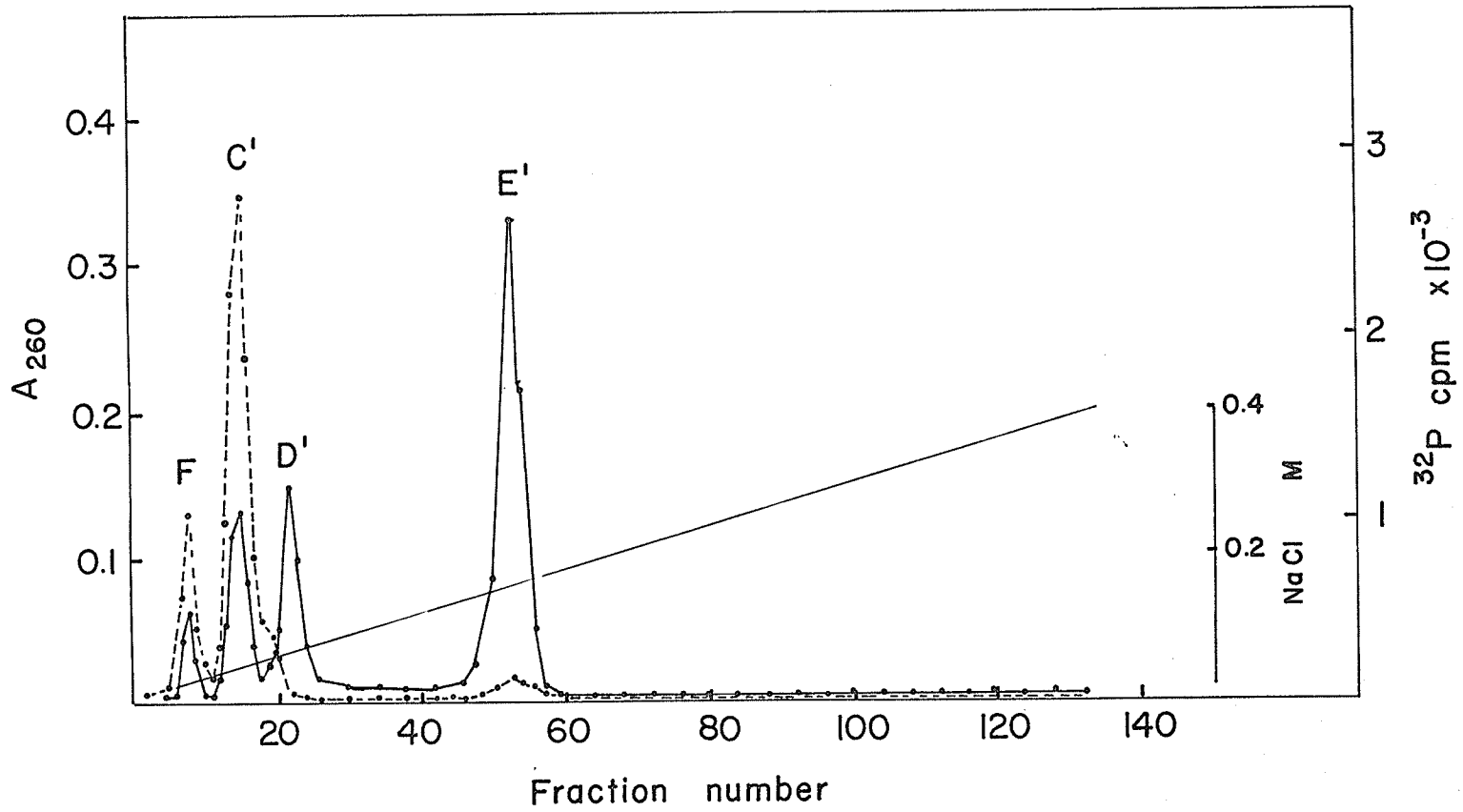

0.4
0.3
0.2
0.1
A_{260}
C'
E'
F
D'
3
2
1
^{32}P cpm $\times 10^{-3}$
0.4
0.2
NaCl M
20
40
60
80
100
120
140
Fraction number

Table 23. Chromatographic analysis of fragments produced by 6 h digestion of HS3 by nucleotide pyrophosphatase.

Fragment or standards	Rf value modified solvent I	solvent III
F	0.79	0.70
C'	0.68	0.20
D'	0.77	0.98
E'	0.21	0.95
AMP	0.69	0.20
5'UMP	0.79	0.70
2'-3'UMP	0.79	0.66
UTP	0.70	0.77
P_i	0.76	0.98

Table 24. Chemical analysis of fragments produced by 6 h digestion of HS3 with nucleotide pyrophosphatase.

Fragment	nmoles		Ratio
	PO_4	base*	base:PO_4
F	16	15.5	1:1
C'	34	33	1:1.1
D'	35	-	
E'	185	18	1:10.3
ΣF+E'	201	33.5	1:6

* Extinction coefficients of 14.5×10^3 for fragment C' and 9.9×10^3 for fragments F and E' were used to calculate nmoles of nucleobase.

on PEI-cellulose, however, on paper in solvent III fragment E' migrated with R_f 0.95 (table 23). Analysis of fragment E' showed it to contain the amino acid glutamate which was only observable after acid hydrolysis. Fragment E' has a base to phosphate ratio of 1:10.3 (table 24). This is much higher than that found for fragment E (figure 23). But by taking the sum of the phosphate and base for fragments E'and F, one obtains a base to phosphate ratio of 1:6. This suggests that fragment F is hydrolyzed from fragment E. Analysis of the phosphate in fragment F shows the equivalent of about ½ a phosphate (0.47 of 1 phosphate when compared to fragment C') present. Quantitation of the nucleobase present in fragment F as a ratio of the total in F and E', showed that fragment F contains 46% of the uridine initially computed in fragment E. This is released as UMP by nucleotide pyrophosphatase.

Therefore nucleotide pyrophosphatase digests HS3, in 4 h, into AMP, XP_i, and UDP-mannitol tetraphosphate (glutamate*). After 6 h digestion some UMP is released. Fragment E' then must contain the remaining undigested UDP-mannitol tetraphosphate

(glutamate*) plus the product produced after UMP release, which can not be differentiated on the basis of three chromatographic systems.

The amino acid glutamate seems to be associated with either the ADP-X fragment (produced by KOH hydrolysis) or the UDP-mannitol tetraphosphate fragment produced on nucleotide pyrophosphatase digestion. UMP which is released by prolonged digestion of the UDP-mannitol tetraphosphate(glutamate*) did not contain glutamate, suggesting that glutamate* is not attached to the uridine monophosphate unit.

D. ANALYSIS OF MAMMALIAN HS3

Much smaller yields of mammalian HS3 were obtained in comparison to that obtained from Achlya, due to the cost and problems in growing up large populations of cells. For these reasons, the analysis of mammalian cell HS3 was limited. Mammalian HS3 was analyzed, however, for various components present in Achlya HS3 to determine whether or not the fungal and mammalian cell HS3 were identical.

1. Spectral Analysis

Mammalian HS3 was spectrally analyzed between A_{220} and A_{300} nm at pH 7 (figure 25). The spectral data are summarized in table 25. Mammalian HS3 absorbs maximally at 260nm with the wavelength of minimum absorption at 230nm. The spectral ratios (table 25) show close similarities to that of Achlya HS3.

2. Nucleoside Analysis

The base constituents of mammalian HS3 were determined after digestion of the molecule by a

Figure 25: Spectral scan of mammalian HS3 at pH 7.0.

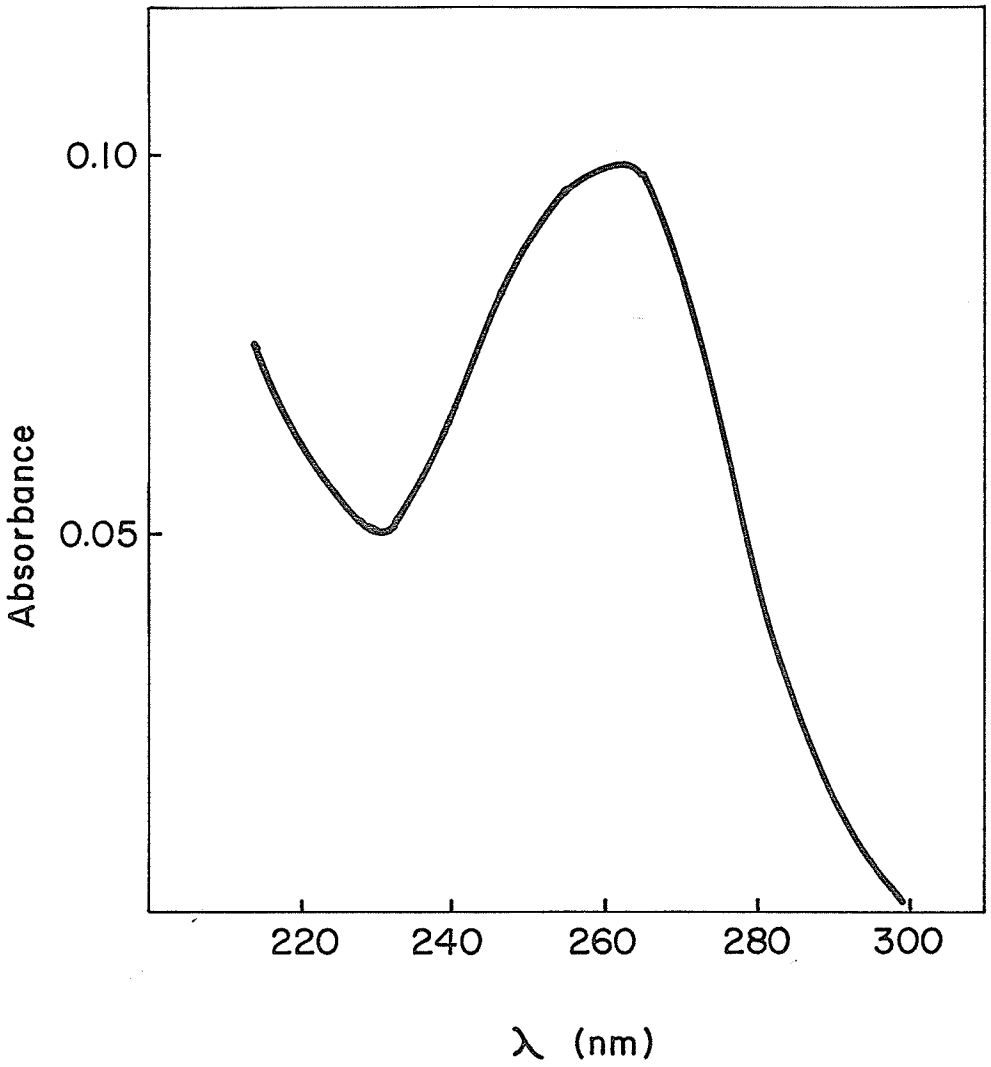

0.10
0.05
Absorbance
220
240
260
280
300
λ (nm)

Table 25. Spectral data for mammalian HS3 at pH 7.0.

λmax	260
λmin	231
Absorbance ratios	
250:260	0.84
280:260	0.45
290:260	0.15

combination of enzymes. HS3 was digested with a mixture of nucleotide pyrophosphatase and bacterial alkaline phosphatase for 2 h (details given in the legend to table 26). As shown in table 26 mammalian HS3 contained two uv-absorbing entities which comigrated in both dimensions with adenosine and uridine. These results indicate that mammalian HS3 contains identical nucleoside constituents, as was found in Achlya HS3.

3. Amino Acid Analysis

After acid hydrolysis of mammalian HS3 (0.5 units at A_{260}) analysis for amino acids was carried out. After two-dimensional thin layer chromatography with solvents VI and VII, ninhydrin spray revealed a single major orange red spot (figure 26) which migrated coincidently with authentic glutamate. These results indicate that mammalian HS3 contains glutamate as does Achlya HS3.

Table 26. Chromatographic properties† of uv-light absorbing products derived from mammalian HS3 degraded by bacterial alkaline phosphatase and nucleotide pyrophosphatase*.

Product	R_f solvent IV	solvent V
A	0.62	0.31
B	0.36	0.76
uridine	0.38	0.76
adenosine	0.64	0.31

* Enzymes used at 1µg protein/A_{260} of compound in 0.2M tris-HCl, pH 7.5 and 0.02M $MgCl_2$. Incubation time, 2 h.

† chromatographed on thin layer cellulose plates.

Figure 26. Amino acid analysis of mammalian HS3. Photograph of the two-dimensional chromatogram showing the amino acid glutamate.

2
1

4. Carbohydrate and Phosphate Analysis

The purine-bound ribose content of mammalian HS3 was determined by orcinol method. Phosphate analysis was carried out as described in Methods. Table 27 shows that mammalian HS3 has a purine-bound ribose to base ratio of 1:1. The purine-bound ribose to phosphate ratio is approximately 1:8, and the base to phosphate ratio was estimated to be approximately 1:4. These ratios agree with those estimated for Achlya HS3 (table 6).

For the determination of pyrimidine-bound ribose the sample was pretreated with 1% sodium amalgam; however, this did not result in any increase in the ribose content (table 28). Mammalian HS3 when acid hydrolyzed for 1 h and reduced with sodium amalgam for 8 h showed a 30-35% increase over the estimated purine-bound ribose. A similar result was obtained for Achlya HS3 (discussed in "Carbohydrate Analysis of Achlya HS3"). The inability to obtain the expected amount (double the purine-bound ribose) of ribose was found to be due to the partial reduction (approximately 75%) of the purine-bound ribose (released by the HCl treatment) to ribitol (ribitol phosphate). The reduced sugar

Table 27. Stoichiometry of some of the components in mammalian HS3.

Compound	nmoles			ratios		
	ribose*	PO_4	base**	ribose:base	base:PO_4	ribose:PO_4
HS3	17	140	33	1:1.9	1:4.2	1:8.2
ATP	21	64	20	1:1.0	1:3.2	1:3.0

* only the purine-bound ribose estimated by the standard orcinol procedure.

** total base was calculated from A_{260} values of acid hydrolyzed products using an average extinction coefficient of 11.5×10^3 per base.

Table 28. Analysis of purine and pyrimidine-bound ribose in mammalian HS3

sample	sodium amalgam reduction (8h)	ribose nmoles
HS3	-	17
HS3	+	17.5
*acid hydrolyzed HS3	+	23

* acid hydrolyzed samples treated with 1N HCl for 1 hour at 105°. The acid was removed *in vacuo*, and the sample resuspended in deionized water.

is not estimable by the orcinol proceedure, such that only 25% of the purine-bound ribose was estimated. Based on the above percentage, the results indicate that mammalian HS3 contains a pyrimidine-bound ribose to purine-bound ribose ratio of 1:1.

E. ANALYSIS OF ACHLYA HS2 AND HS1

1. Spectral Analysis

Purified HS2 and 1 were spectrally analyzed between λ_{220} and λ_{300} nm at pH 1, 7 and 12 and the data are summarized in table 29. The spectral data of uridine is also included. Spectral scans (at pH 7) for HS2 and HS1 are shown in figures 27 and 28 respectively. HS2 and 1 absorb maximally around 260 at each pH value with the wavelength of minimum absorption shifting at alkaline pH. This behaviour is characteristic of uracil and uracil containing nucleotides. The shift in the minimum exhibited by HS2 and HS1(from 232 to 240 and 245nm respectively) is much more extreme than that displayed by HS3 (table 2).

The uv-light absorbance characteristic displayed by HS2 and 1 is sensitive to increasing salt concentrations similar to what has been shown for HS3. Both HS2 and 1 absorb maximally in distilled water. The uv-light absorbance of HS2 at 260nm showed a hyperchromic effect of 25% during incub-

Table 29. Spectral data of HS2 and HS1

compound	pH	nm max	nm min	Absorbance ratios 250:260	280:260	290:260
HS2	1	260	230	0.78	0.40	0.08
	7	260	230	0.76	0.39	0.06
	12	260	240	0.84	0.32	0.03
HS1	1	260	231	0.82	0.39	0.10
	7	261	233	0.86	0.37	0.10
	12	260	245	0.89	0.35	0.09
uridine*	1-7	262	231	0.74	0.35	0.03
	12	262	242	0.83	0.29	0.02

* data for uridine obtained from Burton (1969).

Figure 27: Spectral scan of HS2 at pH 7.0.

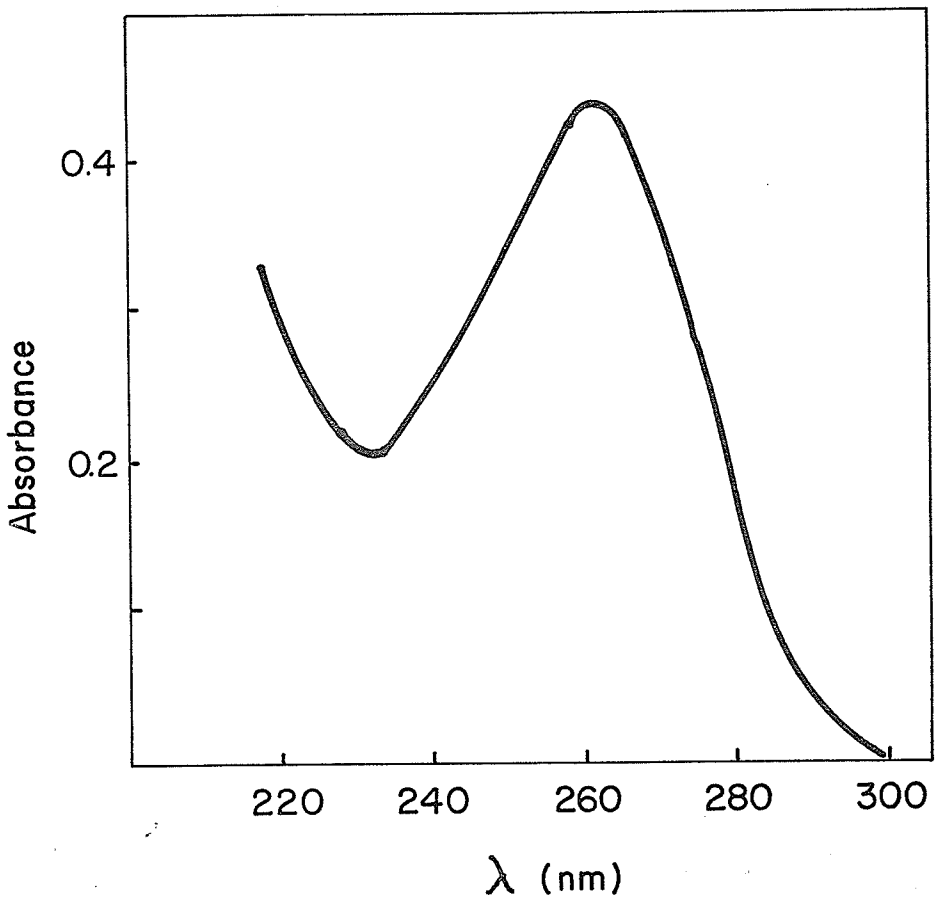
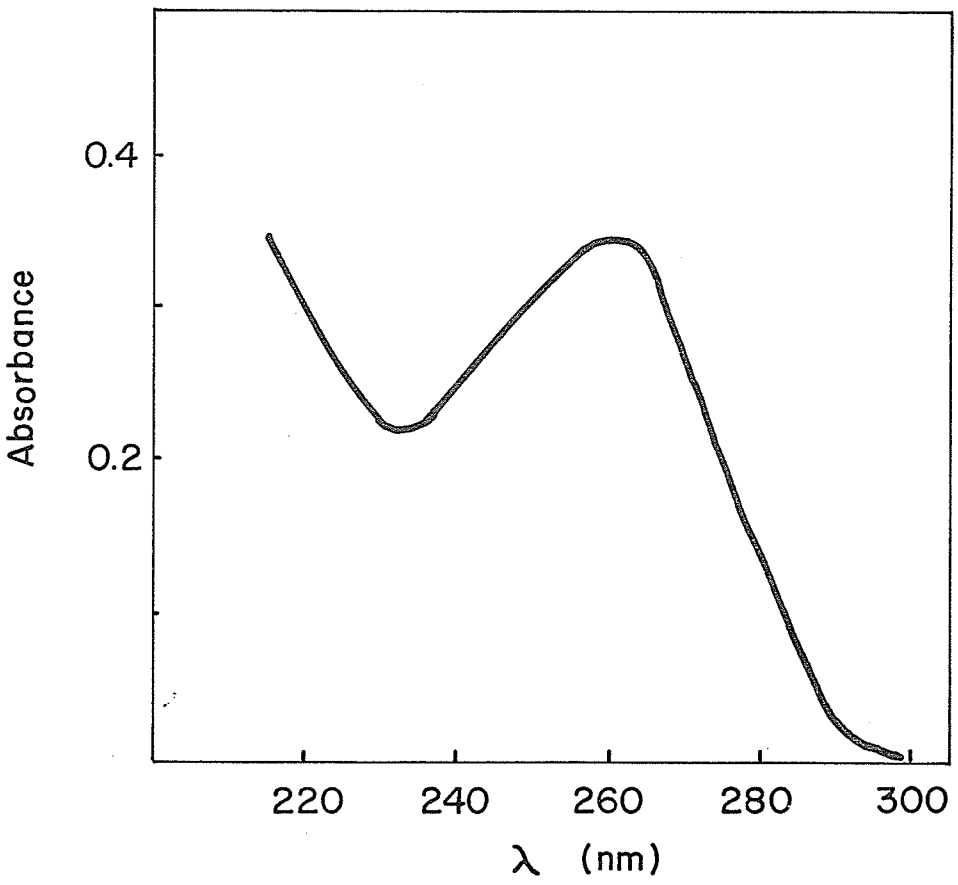

0.4
0.2
Absorbance
220
240
260
280
300
λ (nm)

Figure 28: Spectral scan of HS1 at pH 7.0.


0.4
0.2
Absorbance
220
240
260
280
300
λ (nm)

ation with 0.33N KOH at 37° for 30 min. An increase of approximately 50% in the uv-light absorbance at 260nm was observed during treatment of HS2 and HS1 (some preparations showed as much as 100% increase) with 1N HCL (1 h at 100°), a treatment which degrades the molecule.

2. Nucleoside Analysis

^{32}P-labelled HS2 and 1 were acid hydrolyzed *in vacuo* for 1 h and 20 h in 1N HCl at 105°. The products were chromatographed on paper in solvent III (table 30). After 1 h hydrolysis, HS2 and HS1 released 1 uv-light absorbing entity which migrated with UMP and contained ^{32}P-radioactivity. The remainder of the ^{32}P-radioactivity migrated with the solvent front (R_f 0.95). After acid hydrolysis for 20 h, both HS2 and HS1 released one uv-light absorbing entity that migrated with uridine.

The nucleoside constituents of ^{32}P-labelled HS2 and HS1 were also determined after digestion of the molecule by a combination of nucleotide pyrophosphatase and bacterial alkaline phosphatase

Table 30. Chromatographic analysis of uv-light absorbing products released on acid hydrolysis of HS2 and HS1.

compound	R_f values of products after acid hydrolysis for 1 hour	20 hours
HS2	0.70	0.56
HS1	0.70	0.56
UMP+	0.70	
uridine+	0.56	
uracil+	0.54	

* chromatographed on paper in solvent III

\+ standards

for 2 h. The uv-light absorbing products were separated two-dimensionally. Authentic uridine was used as a marker. As shown in table 31, both HS2 and HS1 contained a uv-absorbing entity which comigrated in both dimensions with uridine. Estimation of the nucleobase content of HS2 and HS1 revealed that both these compounds contained a base to ribose ratio of 2:1 (table 32).

3. Carbohydrate Analysis

Chromatographic identification of the sugars present in HS2 (as described in methods) revealed that HS2 contained two sugars, one of which was ribose, and another, exhibiting the same properties as the sugar designated 'Y' (mannitol) present in HS3 (see table 10 for clarification). HS2 was also found to be labelled with ^{14}C-mannitol (see figure 15 in "Carbohydrate Analysis of HS3"). The ^{14}C-radioactivity in HS2 was found to be associated with mannitol as was shown for HS3. These results suggest that HS2 contains both mannitol and ribose.

Table 31. Chromatographic analysis† of the uv-light absorbing products derived from HS2 and HS1 degraded by bacterial alkaline phosphatase and nucleotide pyrophosphatase+.

Compound	R_f solvent IV	solvent VI
HS2	0.38	0.76
HS1	0.38	0.76
uridine*	0.38	0.76

+ Enzymes used at 1µg protein/A_{260} of compound in 0.2M tris-HCl, pH 7.5 anf 0.02M $MgCl_2$. Incubated at 37^{o} for 2 h.

* standard

† chromatographed on thin layer cellulose plates.

Table 32. Stoichiometry of some of the identifiable components of HS2 and HS1.

	nmoles			ratios		
Compound	ribose*	PO_4^-	base**	ribose:base	ribose:PO_4	base:PO_4
HS2	8	75	17	1:2.1	1:9.3	1:4.3
HS1	6	72	12	1:2	1:12	1:6.2

* ribose as estimated by the standard orcinol procedure.

**total base was calculated from A_{260} values of acid hydrolyzed samples, (1N HCl for 1 h at 105°) with an extinction coefficient of 9.9×10^3.

HS2 (and HS1) was found to contain one ribose per molecule that was estimable by the standard orcinol procedure (table 32). Analysis of HS2 for pyrimidine-bound ribose (pretreatment with 1% sodium amalgam) only resulted in a 40% increase in estimable ribose. The pyrimidine-bound ribose in HS2 was then estimated (by the orcinol procedure) after acid hydrolysis (1N HCl for 1 h at 105^{o}) followed by sodium amalgam reduction (12 h). The results are shown in table 33. A total of 14 nmoles of pyrimidine-bound ribose was estimated. The ribose estimated by the standard orcinol procedure (table 32) was not reactive during the pyrimidine-bound ribose quantitation due to its release by acid and conversion to ribitol or ribitol phosphate by sodium amalgam reduction (Pigman and Goepp 1948). Authentic ribose (10-20 nmoles) was completely reduced after 12 h at room temperature by 1% sodium amalgam showing no reaction with orcinol. These results suggest that HS2 contains 3 moles of ribose per molecule, two of which are pyrimidine-bound. Hydrogenation of uridine is considerably retarded due to the intramolecular interaction between the

heterocyclic base and sugar residue (Kochetkov et al. 1971). The inability to estimate the pyrimidine-bound ribose in HS2 after sodium amalgam reduction suggests the reduction process was ineffective and this would suggest that there are uridine dinucleotide sugar components in HS2.

Quantitation of the amount of mannitol in HS2 was carried out using the procedure of Lambert and Neich (1950), which measures the formaldehyde released on periodate oxidation of terminal alcoholic groups (CH_2OH). Aliquots of HS2, acid hydrolyzed for 20 h with 1N HCl at 105° in vacuo were analyzed. The results are shown in table 33.
HS2 was found to contain approximately 1 mole mannitol per molecule of HS2 (see table 33 legend for method of calculating nmoles mannitol).

These results suggest HS2 to contain 3 moles ribose and 1 mole mannitol per molecule of HS2.

4. Phosphate Analysis

Total phosphate was determined after ashing as described in Methods, and correlated with ribose (as estimated by the standard orcinol procedure)

Table 33. Evaluation and stoichiometry of the carbohydrate content of HS2

compound	nmoles	Ratio compound:base
ribose*	7	1:1.9
pyrimidine-bound ribose	14	1:1
formaldehyde (mannitol+)	32(5.5)	1:2.4(mannitol)
base**	13.5	1:1

* ribose estimated by the standard orcinol procedure

** total base estimated on acid hydrolyzed samples (1N HCl for 1 h at 105^{o}) using an extinction coefficient of 9.9×10^{3}.

\+ mannitol estimated from the formaldehyde determined (32 nmoles formaldehyde determined of which 21 came from ribose. Therefore, 32-21 or 11 nmoles formaldehyde released from mannitol. Since mannitol releases two moles formaldehyde per mole, there is 11/2 or 5.5 nmoles mannitol).

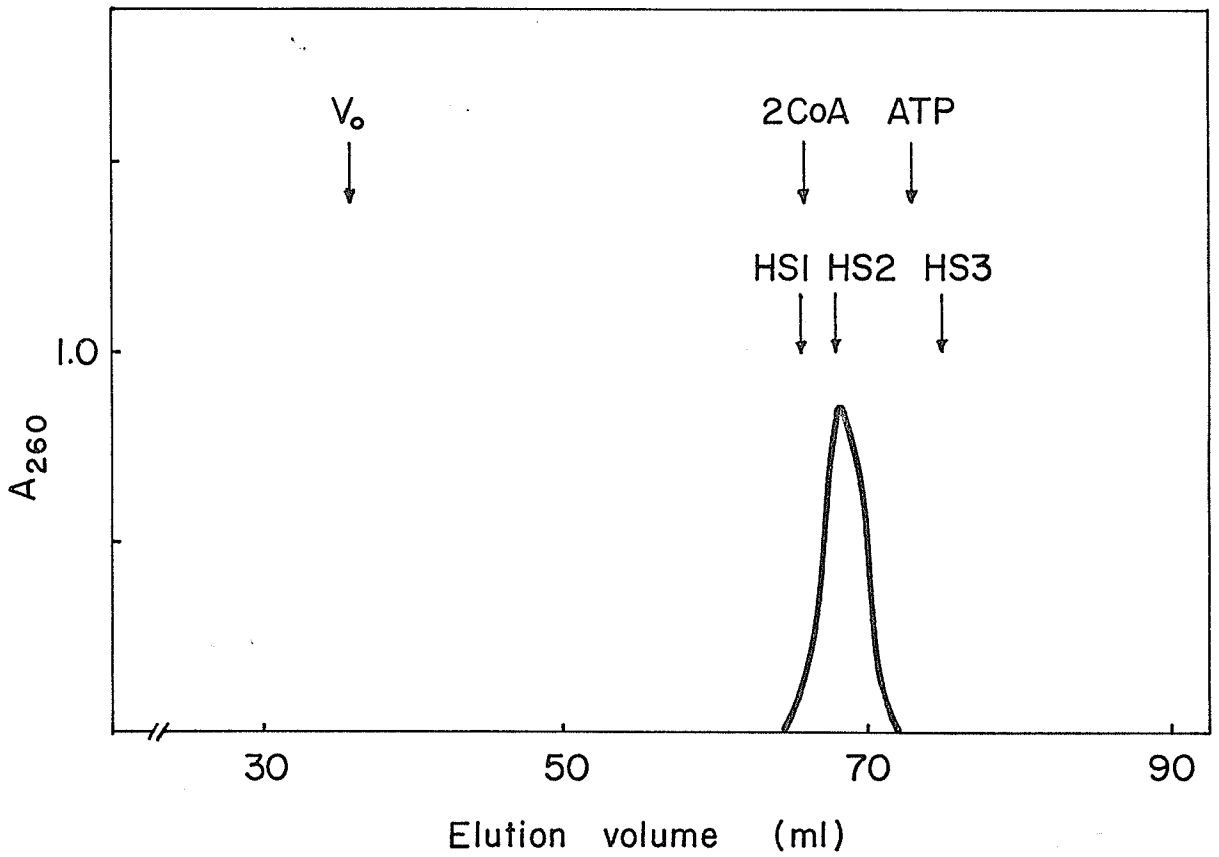
and nucleobase content. The results shown in table 32 indicate that HS2 has 9 or 10 phosphates and HS1 has 12 phosphates per molecule.

5. Sephadex G50 Chromatography

HS2 is a large molecule containing two uridines, ribose, mannitol and approximately 9-10 phosphates. HS1 has been shown to contain two uridines, ribose and 12 phosphates. Neither HS2 nor HS1 were found to contain any amino acids. Figure 29 shows the elution profile of HS2 chromatographed on Sephadex G50. HS2 eluted in 68 ml. HS1 eluted from the column in 66 ml. The order of elution of the three HS compounds is HS1, HS2 and HS3.

A molecular weight calculation of HS2 based on a summation of the individual component molecular weights, indicates that HS2 has a minimum molecular weight of approximately 1720-1820 daltons. HS2 and HS3 have similar molecular weights (HS3, 1792 daltons), however, these two compounds elute in different volumes on Sephadex G50 chromatography. This suggests that the two compounds might have different molecular weights. This could be explained by the

Figure 29: Chromatography of HS2 and HS1 on Sephadex G-50 preequilibrated with 50mM TEAB buffer. For details see Methods.


V_o
2CoA
ATP
HS1
HS2
HS3
1.0
A_{260}
30
50
70
90
Elution volume (ml)

presence of another as yet unidentified component in HS2 which might account for the suggested apparent difference in molecular weight, or HS2 might have a different molecular structure (tertiary structure) such that it has a larger molecular size than HS3 which results in a smaller elution volume.

6. Stability

Very little data has been accumulated on the stability of HS2 and 1. These compounds are however, unstable in acid and in alkali. Storage of these compounds for prolonged periods (up to 2 months) at -20^{o} in water (pH 5.0) did not result in any decrease in their inhibitory activity on RNA and DNA polymerases. Rechromatography on Sephadex-A25 at pH 3.6 of month old preparations indicated the compounds were still intact. However, these same preparations were unstable to rechromatography on PEI-cellulose.

DISCUSSION

DISCUSSION

This study was concerned with the isolation, purification and chemical, physical and enzymatic analysis of three polyphosphorylated dinucleosides HS3, HS2 and HS1. The following sections will summarize and discuss the results of this investigation, the purpose of which was to chemically define these three unusual compounds. That goal was only partly reached.

HS3

HS3 has been found to occur in a variety of fungi (LéJohn et al. 1975) and various mammalian cell lines grown in culture (Goh and LéJohn 1977). While much of the chemical constituents and their organization in HS3 has been determined, there remain a few points of uncertainty that need to be discussed. But first a summary of what is known about the components and their molecular organization in HS3 is pertinent.

HS3 was found to be a composite of different

classes of smaller molecules. For example, there are nucleosides (adenosine and uridine), D-glutamate, two monosaccharides, mannitol and sugar X (an exact idenitification of sugar X cannot be made based on the available data which is discussed below) and phosphates. The mole ratio of these components was found to be one of adenosine, uridine, glutamate, mannitol and sugar X and 8 of phosphate per molecule of HS3. A summation of the molecular weights of the components, indicates that HS3 has a minimum molecular weight of approximately 1792 daltons assuming sugar X is ribitol.

Sugar X was found to be a monosaccharide of the amino polyol type based on its reactivity with specific indicator sprays (table 10). It chromatographed on paper very near ribitol in three solvent systems used (table 10). Chemical analysis of sugar X showed it to have a five carbon structure with a ninhydrin reacting amino substituent probably attached to a terminal carbon. Based on these data one can speculate that sugar X may be an amino derivative of ribitol; however, the exact definition of sugar X will have to await

a more complete chemical (melting point) and spectral (nuclear magnetic resonance and infra-red spectroscopy) analysis, techniques which require large quantities for analysis. This luxury is beyond our capabilities at the moment.

Experiments aimed at elucidation of the molecular organization of the components in HS3 are summarized in figure 17. KOH degraded HS3 into two fragments (A and B, figure 18) by hydrolyzing a sensitive carboxyphospho anhydride bond (table 11). Analysis of fragment A gave the following results.

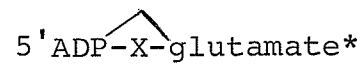
(1) fragment A contained one mole of adenosine, sugar X and 2 moles of phosphate. It cochromatographed on paper and on thin layer cellulose plates with authentic ADP. Fragment A also contained D-glutamate which was only recovered after hydrolysis in acid or strong KOH.

(2) fragment A was digested by nucleotide pyrophosphatase (and snake venom phosphodiesterase) into 5'AMP (subfragment A1) and XP_i (subfragment A2). Neither A1 nor A2, however, contained D-glutamate. D-glutamate was only detected (as analyzed by two dimensional chromatography and ninhydrin reactivity)

in the unfractionated nucleotide pyrophosphatase digestion mixture of HS3 followed by acid hydrolysis. These results suggest that glutamate must be released (since it is not found associated with either subfragment) in a modified form (designated glutamate*) which is ninhydrin negative but is converted to D-glutamate by acid or KOH hydrolysis. The cleavage of fragment A by nucleotide pyrophosphatase suggests that the phosphates are in pyrophosphate linkages. Since subfragment A1 was found to be 5'AMP, it would seem that the pyrophosphate group is localized at the 5' position of adenosine. The attachment of the pyrophosphate group to the 5' position is also suggested by the ability of snake venom phosphodiesterase (requires free 3'OH ribose) to cleave fragment A. Thus, fragment A contains ADP.

Subfragment A2 was found to contain XP_i, indicating that sugar X is probably bound to the terminal phosphate of the ADP moiety. The phosphate in A2 (XP_i) may be linked to the terminal CH_2OH group on sugar X as acid hydrolysis was required before this group was estimable as formaldehyde.

One might speculate that the amino substituent on sugar X (see page 162) is attached to the other terminal carbon in subfragment A2 as prolonged acid hydrolysis of A2 released another CH_2OH group estimated as formaldehyde. After acid treatment of XP_i no ninhydrin-reactive component was detectable. (3) When fragment A was subjected to limited acid hydrolysis subfragments A3, A4 and A5 were obtained (figure 21). A3 was identified as adenine, A4 as ribose phosphate and A5 contained XP_i (glutamate). These results suggest that glutamate* is attached to either the terminal phosphate or to sugar X These two possibilities will be considered later. The above results suggest that fragment A might be



Analysis of fragment B gave the following results.

(1) Fragment B contained one mole of uridine and mannitol and six moles of phosphate.

(2) Fragment B was digested very slowly by nucleotide pyrophosphatase releasing UMP suggesting that it is UDP. Bovine spleen phosphodiesterase (requires

a free 5'OH ribose group for activity), however, did not digest fragment B. This indicates that at least one phosphate is located on the 5'OH position of uridine.

(3) Bacterial alkaline phosphatase did not release any P_i from fragment B suggesting that the phosphates might not be accessible to phosphomonoesterase action.

(4) Kochetkov (1964, 1972) has shown that UDP-sugar compounds are resistant to hydrogenation in comparison to the common uridine nucleotides due to an intramolecular interaction between the carbohydrate and the heterocyclic base in the UDP-sugar molecule. Since fragment B displayed this characteristic (inability to estimate ribose after prolonged treatment with sodium amalgam) and it contains mannitol, this suggests fragment B might be UDP-mannitol with 4 phosphates attached to it forming UDP-mannitol tetraphosphate. Fragments A and B may be covalently linked by a carboxy-phospho anhydride bond.

Nucleotide pyrophosphatase digestion of HS3 for 4 h released three fragments (C, D and E,

figure 23). Analysis showed that fragment C was 5'AMP and D,XP_i. Fragment E contained one mole of uridine and mannitol and six moles of phosphates. Fragment E contained glutamate which was released by acid hydrolysis. Six hours digestion of HS3 with nucleotide pyrophosphatase released four fragments (figure 24) designated C', D', E' and F. Fragments C' and D' are identical to C and D of the 4 h digestion experiment (figure 23). Fragment F is UMP. Phosphate and nucleobase estimation indicated that this product arose from enzymic action on fragment E' (or E of figure 23). Fragment E' then is probably a mixture of undigested UDP-mannitol tetraphosphate and mannitol pentaphosphate. Only fragment E' contained glutamate.

Nucleotide pyrophosphatase digests HS3 such that glutamate* is attached to the UDP-mannitol tetraphosphate moiety. KOH hydrolyzed a carboxy-phospho anhydride linkage in HS3 such that glutamate* was attached to the ADP-X moiety. These results indicate that glutamate probably links covalently the ADP-X and the UDP-mannitol tetraphosphate moieties of HS3, and the mode of attachment of

glutamate* to the UDP-mannitol tetraphosphate moiety is by a carboxyphospho anhydride bond. Since glutamate was not associated with UMP (fragment F) the carboxyphospho anhydride linkage could occur between the carboxyl group on glutamate* and a phosphate group attached to the mannitol sugar; however, this is purely speculative.

In summary, HS3 appears to be ADP-X(glutamate) covalently bonded to UDP-mannitol tetraphosphate by a carboxyphospho anhydride group involving glutamate and a phosphate on the UDP-mannitol tetraphosphate moiety. At this stage, the structure could be written as

$$\overset{\frown}{\text{ADP-X-(glu*)}}\text{:(4P}_i\text{) mannitol-UDP}$$

The uncertainties with respect to the molecular organization of HS3 are as follows. To begin with, the manner with which the four phosphates associated with the UDP-mannitol are arranged is unresolved. These phosphates are not removed as inorganic phosphate either with nucleotide pyrophosphatase or bacterial alkaline phosphatase. Nor does periodate

oxidation of fragment B cause release of any phosphorylated compounds which would suggest that there are no free adjacent hydroxyl groups that are cleavable by periodate on the mannitol sugar.

Secondly, the molecular configuration of D-glutamate in HS3 is enigmatic for D-glutamate is only recovered after acid hydrolysis not by enzyme digestion nor boiling in H_2O for 20 h in vacuo. Glutamate appeared to be released from fragment A after nucleotide pyrophosphatase digestion although how this occurs is not understood. In the intact molecule, the amino group cannot react with ninhydrin which could mean it is involved in some form of covalent linkage. Acid hydrolysis then probably breaks glutamate* releasing glutamate as was observed.

Thirdly, the molecular linkage between the ADP-X moiety and glutamate* is unknown. Glutamate was associated with sugar XP_i (subfragment A5) which indicates that it is either bound to sugar X or the phosphate. One might speculate that glutamate* could be linked through the amino substituent on sugar X, based on the observation that fragment A

was ninhydrin negative although it contains the amino substituent on sugar X (and on glutamate*). If the amino substituent on sugar X was involved in a covalent linkage one could not expect it to react with ninhydrin. Of course it is possible that failure of fragment A to react with ninhydrin could just as well be due to its molecular conformation such that the amino substituent is protected.

The uncertainties discussed are not unsolvable, but to do so would require much greater quantities of the compound. The elucidation of the components and partial structure of HS3, however, has given an insight into studies on possible enzymes that may be used for its synthesis and degradation (in vivo). A study of these enzymes and the pathway of synthesis involved might aid in elucidating the complete molecular structure of HS3.

Mammalian HS3 (isolated from Chinese hamster ovary cells) was analyzed for various properties and components found in Achlya HS3. The data are summarized in table 34. Mammalian HS3 cochromatographed with Achlya HS3 two dimensionally on thin layer plates and on DEAE-Sephadex A25 at pH 3.6 and 8.0.

Table 34. Comparison of the properties of Achlya and mammalian HS3 (modified from Lewis et al. 1977).

Property	Achlya HS3	Mammalian HS3
R_f [1]	0.22(0.35)	0.21(0.36)
DEAE-Sephadex-A25 elution at:		
(i) pH 8.0	1.1M	1.1M
(ii) pH 3.6	0.28M	0.27M
λmax at pH 7.0	260nm	260nm
λmin at pH 7.0	232nm	231nm
$A_{250/260}$ at pH 7.0	0.84	0.84
$A_{280/260}$ at pH 7.0	0.45	0.41
$A_{290/260}$ at pH 7.0	0.16	0.16
Resistance to alkaline phosphatase*	resistant	resistant
Combined treatment with phosphatase and pyrophosphatase	degraded	degraded
Components		
adenosine	+	+
uridine	+	+
glutamate	+	+
8 phosphates	+	+
mannitol	+	N.T.
sugar X	+	N.T.

[1] Two dimensional chromatography on PEI-cellulose in solvent II (first dimension) and solvent I (second dimension).

* Incubated at 37° for 1 h with bacterial alkaline phosphatase.

+ Present, N.T.-not tested.

Mammalian HS3 displayed similar spectral characteristics as Achlya HS3. Mammalian HS3 was degraded to adenosine and uridine by the combined treatment of bacterial alkaline phosphatase and nucleotide pyrophosphatase. These products were also released in a similar manner from Achlya HS3. Mammalian HS3 contains adenosine, uridine, a purine-bound ribose, a pyrimidine-bound ribose and 8 phosphates per molecule. Mammalian HS3 was also shown to contain glutamate but this component was not quantitated stoichiometrically.

Thus mammalian HS3 contains the same mole ratio of components as those found in Achlya HS3 (analysis for carbohydrates in mammalian HS3 was not carried out due to insufficient quantities). Based on these data it was concluded that mammalian HS3 is probably identical to Achlya HS3. This conclusion is also supported by enzyme inhibition studies. Lewis et al. (1977) have shown that HS3 purified from either Achlya or CHO cells inhibited ribonucleotide reductases isolated from CHO cells. Achlya HS3 also inhibited the enzyme isolated from

Achlya (Lewis et al. 1976). Both mammalian HS3 and Achlya HS3 were found to be equally potent in inhibiting DNA-dependent RNA polymerases isolated from both Achlya and CHO cells (G.R.Klassen, R.D.McNaughton, I.Susuki and H.B.LéJohn (1977), Proceedings of the Canadian Federation of Biological Societies, Vol. 20 pp 21). These data support the conclusion that Achlya HS3 andsmammalian HS3 are probably identical.

HS2 and HS1

HS2 was found to contain 1 mole of mannitol and ribose, 2 moles of uridine and 9-10 phosphates per molecule. The two pyrimidine-bound ribose in HS2 were not estimable after hydrogenation of intact HS2 with sodium amalgam for prolonged periods. Kochetkov et al. (1964, 1972) have shown that uridine nucleotide sugars molecules are resistant to hydrogentation when compared to common uridine nucleotides. This property which is exhibited by HS2 may suggest that both uridines in HS2 are in the form of uridine diphosphate sugar moieties.

This is supported by the fact that HS2 contains ribose and mannitol. Since HS2 contains mannitol and uridine which is also found in HS3 one might speculate that HS2 contains the UDP-mannitol tetraphosphate moiety found in HS3. The molecular organization of the components in HS2, however, will have to await future studies.

HS1 was found to contain one mole of ribose, 2 moles of uridine and 12 phosphates per molecule. Analysis for mannitol was not carried out due to limited amount of available material. HS1 displayed similar spectral characteristics as HS2. HS1 was degraded by a mixture of nucleotide pyrophosphatase and bacterial alkaline phosphatase releasing uridine as was found with HS2. HS1 eluted very close to HS2 on Sephadex-G50 suggesting similar molecular size and structure as HS2. On the surface, it appears as if HS1 is identical to HS2 except that it contains 2 extra phosphates. This may not be the case, however, as HS1 (but not HS2) was found to inhibit an unresolved mixture of DNA polymerases isolated from Achlya (LéJohn et al. 1978).

In summary HS3, 2 and 1 are all highly phosphorylated dinucleoside sugar compounds

unlike anyyhitherto discovered (see Historical). Only HPNIII from B. subtilis (Rhaese et al. 1975) with the reported tentative structure ppZpUp (where Z is an undefined sugar) bears some resemblance to fragment B produced by KOH hydrolysis of HS3. But as a chemical analysis of HPNIII has not been reported,this similarity is in doubt.

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