SPERMIDINE, SPERMINE, AND RELATED AMINES

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The polyamines, *spermidine* and *spermine*, are nonprotein nitrogenous bases that are widely distributed in natural materials. Although these compounds were discovered many years ago, they have been relatively unfamiliar to most investigators. Because they had no striking acute pharmacological effects, and because the significance of their presence was not known, little research was directed toward this area. In recent years several observations have caused renewed interest in these compounds. Studies on the role of the polyamines as growth factors for various microorganisms indicated that they might have a wide range of biological significance. This was supported by analytical studies showing the occurrence of polyamines in many bacteria, plants, and bacteriophages, in addition to animal

tissues. In our laboratory, studies in this field were particularly stimulated by the finding that spermine causes striking damage to the renal tubules.

Especially interesting are the recent studies on the stabilizing effects of the polyamines *in vitro* on various cellular and subcellular components and on nucleic acids. Many of the results obtained can be explained by considering the polyamines as polyvalent cations; indeed some of the effects of the polyamines are comparable to those observed with higher concentrations of Ca^{++} or Mg^{++}. The polyamines, however, because of their polybasic nature, have a particularly high affinity for the cellular polyanions, and thus have more pronounced effects at rather low concentrations. Although all of these statements are based on experiments *in vitro*, it seems likely that these stabilizing effects are important aspects of the physiological functions of the polyamines.

In this review we shall attempt to summarize our present knowledge of spermidine and spermine. Frequent reference will also be made to the closely related diamines, 1,3-diaminopropane, 1,4-diaminobutane (putrescine), and 1,5-diaminopentane (cadaverine). The material reviewed encompasses a wide variety of experimental areas. Although there is still no unifying hypothesis to correlate all the experimental findings, collectively these observations indicate the potential significance of these amines.

The structures of these amines are:

$NH_2(CH_2)_3NH_2$	1,3-Diaminopropane
$NH_2(CH_2)_4NH_2$	1,4-Diaminobutane (putrescine)
$NH_2(CH_2)_5NH_2$	1,5-Diaminopentane (cadaverine)
$NH_2(CH_2)_3NH(CH_2)_4NH_2$	Spermidine
$NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$	Spermine

The earliest publication on spermine is probably the famous letter of Lewenhoeck to the Royal Society in 1677, in which he first described spermatozoa (257). In this letter he also reported: "*Et cum praedicta materia paucillum temporis steterat, in ea observabantur trilaterales figurae ab utraque parte in aculeum desinentes, quibusdam longitudo minutissimae arenae, aliquae aliquantulum majores, ut fig. A. Praeterea, adeo nitidae ac pellucidae, ac si crystallinae fuissent.*"[1] These crystals were presumably spermine phosphate (340).

Subsequently, similar crystals were described by Vauquelin (442), Boettcher (51), Charcot and Robin (71), Schreiner (362) and numerous other investigators. These crystals were described in human semen and in other body fluids obtained from both normal and diseased individuals, as well as in tissue specimens, particularly after storage. The chemical nature of the crystals was unclear, and several erroneous possibilities were presented. Various names were applied to the

[1] *The observations of Mr. Anthoni Lewenhoeck on animalcules engendered in the semen* ". . . When this matter had stood a little while, some three-sided bodies were seen in it, terminating at either end in a point; some were of the length of the smallest grain of sand, and some were a little bigger, as in Fig. A. They were further as bright and clear as if they had been crystals."

crystals,[2] including "Böttcher's crystals," "Charcot-Leyden crystals,"[3] and "Charcot-Robin crystals." The term "spermine" was not applied to these crystals until 1888 (249).

Schreiner (362) was the first to identify the crystals correctly as the phosphate of an organic base, and to show, by crystallographic observations, that the crystals from semen were identical with those from tissues. This was a very thorough study, and included the preparation of the free base and several salts. Despite this, however, most investigators over the next forty years had trouble repeating his observations. Poehl confirmed the isolation of spermine phosphate, but published a number of controversial reports on its reputed therapeutic value; indeed, these led to the marketing of a commercial product of questionable composition and of no clinical value (for references and discussion see references 152, 340, 459). During this period several other investigators also isolated basic compounds from various tissues; these compounds were called "musculamine," "neuridine," and "gerontine," but later studies (106) demonstrated that they were identical with spermine.

In a series of papers published in 1923 to 1927, Dudley, Rosenheim, and Rosenheim (104–108, 339, 340) and Wrede *et al.* (456–461) established the presence of spermine in various mammalian tissues, and determined the correct chemical structure; this structure was confirmed by synthesis. Shortly thereafter (109), spermidine was isolated from ox pancreas, and the postulated structure was also confirmed by synthesis.

The diamines have had a considerably shorter history than spermine. 1,4-Diaminobutane (putrescine) and 1,5-diaminopentane (cadaverine) were first described in decomposing animal material, in cadavers, and in the urine of cystinuric patients. The structures were established by comparison (59, 247, 248, 436) with the synthetic diamines. Subsequently, there were many other reports of the isolation of these diamines from materials that were undergoing bacterial decomposition (for further references see 148).

I. OCCURRENCE OF POLYAMINES IN BIOLOGICAL MATERIALS

A. Polyamines in animal tissues

The highest concentrations of spermidine and spermine in animal tissues are found in pancreas, prostate, and human semen (52, 104, 109, 122, 148, 152, 155, 156, 340, 343, 457). The polyamines in human semen are largely in the seminal plasma, rather than in the spermatozoa, and presumably originate in the pros-

[2] For other references to these older papers see the exhaustive historical reviews of Schreiner in 1878 (362) and of Hämäläinen in 1941 (152).

[3] It is very difficult, however, to be certain of the identity and purity of all of the various crystals isolated by different investigators from different sources, particularly since the descriptions were necessarily mainly histological. The nature of Charcot-Leyden crystals is still not certain; even within the past few years papers have appeared discussing whether these crystals are composed of spermine phosphate (242) or whether they contain some type of protein or polypeptide (23, 183).

tatic secretions (155). The high concentration of spermine in human semen has been utilized for the detection of human semen in several medicolegal studies (35, 114, 119, 120, 316).

Table 1A contains a summary of the analytical studies of Hämäläinen (152) on the spermine concentration in human tissues; the spermine was isolated as the insoluble diflavianate. Table 1B contains a summary of some of the spermidine and spermine analyses obtained in our laboratory by chromatographic procedures. References to miscellaneous other polyamine analyses are included in the footnotes to table 1B. Note that the concentration of the polyamines, as well as the spermidine–spermine ratio, varies considerably from organ to organ and from species to species.

The concentration of diamines is very low in animal tissues, and is accordingly difficult to determine with accuracy or specificity. Several reports, however, have indicated the presence of diamines in animal tissues. *1,3-Diaminopropane* (443) has been found in steer, rat and guinea pig liver, and in human semen. The presence of *1,4-diaminobutane* was reported in the pancreas (91, 122) and liver (443) of several species, in ox lung (122), in bovine (375) and pig brain (206), in human semen (443), and in pupae and caterpillars of the silkworm, *Bombyx mori* (1, 2). The presence of *1,5-diaminopentane* has been reported in liver (477).

Although little or no 1,4-diaminobutane or 1,5-diaminopentane has been detected in the urine of normal subjects, both have been found in the urine of some patients with cystinuria (22, 148, 287, 436). These diamines are thought to result from bacterial decomposition of lysine and arginine. Since large amounts of these amino acids are found in both the urine and the intestinal contents in cystinuria, the diamines could be formed primarily in the urinary tract by contaminating bacteria, or appear there secondarily after intestinal absorption and renal excretion (287).

Although most of the polyamines in animal tissues are present as the free amine, diamidinospermidine (hirudonine) is present in the leech, *Hirudo*

TABLE 1A[1]

Spermine content of some human tissues

Organ	Mean Spermine Concentration (μmol/g wet wt)	Organ	Mean Spermine Concentration (μmol/g wet wt)
Prostate	2.40	Thymus	0.30
Pancreas	0.55	Cerebellum	0.08
Spleen	0.22	Cerebrum	0.05
Liver	0.28	Stomach	0.14
Kidney	0.15	Uterus	0.08
Heart	0.08	Ovary	0.06
Lung	0.08	Salivary gland	0.16
Testis	0.16	Bone marrow	0.73
Thyroid	0.08	Adrenal	0.19
Muscle	0.07	Blood	0.10

[1] The data in table 1A are taken from Hämäläinen (152).

TABLE 1B[1]

Spermidine and spermine content of some animal tissues (μmol per g wet weight)

Organ	Polyamine	Animal					
		Mouse	Rat	Guinea pig	Dog	Man	Bull
Pancreas	Spermidine	2.8	8.6	2.1	2.0		
	Spermine	1.0	1.0	1.4	2.7		
Prostate	Spermidine		7.7		0.2		
	Spermine		5.7		0.8		
Semen	Spermidine					Trace	—
	Spermine					16.3	0.1
Liver	Spermidine	1.4	1.6	0.2			
	Spermine	1.1	1.2	0.4			
Kidney	Spermidine	0.5		0.3			
	Spermine	0.7		0.5			
Testis	Spermidine		0.4				
	Spermine		0.5				
Brain	Spermidine		0.7				
	Spermine		0.2				
Muscle	Spermidine	0.1					
	Spermine	0.2					
Mammary carcinoma	Spermidine	1.2					
	Spermine	0.7					

[1] The data in table 1B are taken from Rosenthal and Tabor (343); in this publication, data are also included showing the presence of polyamines in thymus, lung, stomach, small intestine, large intestine, skin, oviduct, blood leucocytes, and a variety of tumors. Reports from other laboratories using a variety of techniques have shown spermidine in human blood (318), pig brain (206), bovine brain (375), chick embryo (320), and in the pupae and caterpillars of the silkworm *B. mori* (1, 2). Spermine has been found in human blood (318), the muscle and poison of snakes (353, 431), cod roe (339), bovine brain (375), beef heart and skeletal muscle (6), chick embryo (320), in the invertebrates *Cionia intestinalis* (3) and the sea urchin *Echinococcus mirabilis* (309).

medicinalis L (336). Conjugates of spermine and 1,3-diaminopropane with phenolic acids are found in the poisonous secretions of bird spiders, *Pamphobetus tetracanthus* (123). While numerous papers have been published on the occurrence of "malignolipin," a phospholipid conjugate of spermine, in malignant tissues (153, 215–241, 424), several investigators have been unable to confirm its existence (173, 186, 198, 212, 290, 355, 469). Spermidine has been reported to be a principal component of a basic material (mammamine) found in the lactating mammary gland of the rat (244).

B. Polyamines in microorganisms

Polyamines have been reported in a variety of bacteria and yeasts (28, 103, 144, 168, 208, 341, 419, 443). Although there is a wide variation in the amounts found in different microorganisms, some organisms contain rather large quantities of the polyamines. In *Azotobacter vinelandii*, for example (grown on minimal media), the combined value for diamines and triamines (13 μmol per g wet weight) accounts for 22% of the total nitrogen in a protein-free trichloroacetic acid extract and 4% of the total cellular nitrogen (419). More detailed studies have been carried out on *Escherichia coli*; when grown in minimal media, these cells contain, per g wet weight, 15 μmol of 1,4-diaminobutane, 1.5 μmol of spermidine, 0.3 μmol of monoacetyl-1,4-diaminobutane, 0.1 μmol of monoacetylspermidine (both isomers[4]), and 0.5 μmol of a glutathione-like conjugate of spermidine (100, 102, 103, 419). Recently, we have obtained preliminary evidence that *E. coli* also contains approximately 0.3 to 0.7 μmol (per g wet weight) of another incompletely characterized derivative of spermidine that contains several amino acids (92). Some organisms (*e.g.*, *Aspergillus nidulans* and *Saccharomyces cerevisiae*) contain spermine while some (*e.g.*, *S. cerevisiae*, *Neurospora crassa*, *Pasteurella tularensis*, and *A. vinelandii*) contain 1,3-diaminopropane (168, 419, 443). Certain actinomycetes excrete 1,5-diaminopentane and 1-amino-5-hydroxylaminopentane in a bound form [components of succinimycin, ferrioxamine, and ferrimycin (41, 157)]. Many organisms, however, have little or none of the polyamines; *Staphylococcus aureus* cells, for example, contain only trace amounts of 1,4-diaminobutane and no spermidine or spermine (341). Herbst *et al.* (168) showed that, in general, gram-positive organisms contained little or none of the polyamines, while gram-negative organisms contained high concentrations.

The polyamine concentrations of the various bacteria vary with the pH and other cultural conditions (103, 419). It is particularly important to know the composition of the culture medium, since many organisms rapidly take up amines from the medium, and the uptake results in marked changes in the intracellular content of both the free amines and derivatives. It is also likely that the amine concentration in the cells is greater in media containing lysine or arginine (295).

The presence of high concentrations of polyamines in bacteria (*e.g.*, *E. coli*) even when grown on a minimal glucose-salts medium supports the concept that these amines are important biological compounds, rather than just a result of detoxification. This view is based on the generalization that organisms like *E. coli*, when grown on minimal media, do not usually develop biosynthetic pathways, unless the products synthesized are essential for the organism.

C. Polyamines in bacteriophages and other viruses

The concentration of polyamines in bacteriophages varies markedly with the type of bacterial virus studied and with the polyamine composition of the host cell. The concentrations of the polyamines and their derivatives are highest in the T-even type of the *E. coli* bacteriophages (T2, T4, "3"), where they account for

[4] Monoacetylspermidine "A" ($CH_3CONH(CH_2)_4NH(CH_2)_3NH_2$) and monoacetylspermidine "B" ($NH_2(CH_2)_4NH(CH_2)_3NHCOCH_3$).

about 40% of the phage cations (10, 11, 202). In T4, for example, the combined concentration of 1,4-diaminobutane and spermidine is approximately 250 μmol per g dry weight. This is the highest concentration of polyamines observed in any natural material. Isotope studies have shown that these polyamines are derived directly from the polyamines of the host bacteria; there is no evidence that any new polyamine synthesis is induced specifically by the bacteriophage infection. Thus, the amount of each polyamine found in the bacteriophage depends on its concentration in the host bacteria. If, before infection, the polyamine concentration in the host is modified by changes in cultural conditions or by adding an exogenous amine (such as spermine) to the medium, comparable variations are found in the composition of the bacteriophages (10).

The polyamines of the isolated T-even bacteriophages do not exchange with labeled external polyamines, and, thus, their presence in the bacteriophages is not an artifact of the isolation procedure (11). This finding is consistent with previous reports on the relative impermeability of T2, T4, and T6 bacteriophages to other solutes (58, 162). On the other hand, only very low concentrations of polyamines are found in *E. coli* bacteriophage T3 and in *Salmonella typhimurium* phage P22 (10). These bacteriophages are known to be of a permeable type, and the polyamines may have been lost during the isolation, which usually involves several washings with buffers containing Mg^{++}. Support for this explanation is afforded by the low polyamine concentration found in a "permeable" mutant of T4 ("osmotic-mutant") (10, 58). For this reason one cannot state now whether the odd-numbered bacteriophages normally contain polyamines.

Little, if any, polyamine has been found in tobacco mosaic virus, cucumber virus, tomato bushy stunt virus, or polio virus (10, 195). A homolog of spermidine, bis-(3-aminopropyl)-amine, has been described by Johnson and Markham (195) in several plant viruses that contain ribonucleic acid (turnip yellow mosaic, turnip crinkle, broad bean mottle). These plant viruses are the only natural materials in which this triamine has been found. It was not detected in the healthy host of the turnip yellow mosaic virus (Chinese cabbage), and this has led to speculation that the amine is formed in response to the virus infection. It is possible, however, that the amine is present in the host in such low amounts that it cannot be detected until the amine is concentrated by the virus.

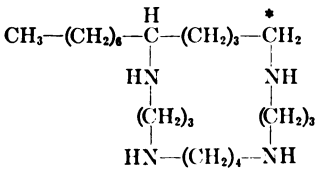
D. Polyamines in plants and in plant alkaloids

Relatively little work has been reported on the distribution of polyamines in plant tissues. 1,4-Diaminobutane has been described in *Datura stramonium*, *Atropa belladonna*, orange juice, barley seedlings, Chinese cabbage, mushrooms, and various other plants (148, 166, 167, 181, 262, 321, 386, 388, 399). This diamine accumulates in considerable amounts in potassium-deficient plants (81, 82, 330, 386, 388), and in broad bean leaves when the plants are maintained in a medium high in NaCl (395). Tetramethyl-1,4-diaminobutane has been isolated from two *Hyoscyamus* species (213, 453). Spermidine has been found in specimens of Chinese cabbage analyzed in our laboratory (399), although, as mentioned above, no triamines were detected in the specimens tested by Johnson

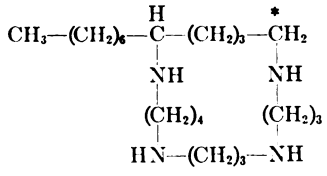
and Markham (195). In unpublished studies with Rosenthal we have also found spermidine in tomato leaves.

Polyamines have recently been described as major constituents of three plant alkaloids:

1. *Pithecolobine*, $C_{22}H_{46}N_4O_2$, has been isolated by Wiesner *et al.* from the bark of the tropical tree, *Pithecolobium saman* Benth. For structural studies pithecolobine has been reduced to deoxypithecolobine, and the following two structures have been proposed for the reduced compound (310, 450–452):



I

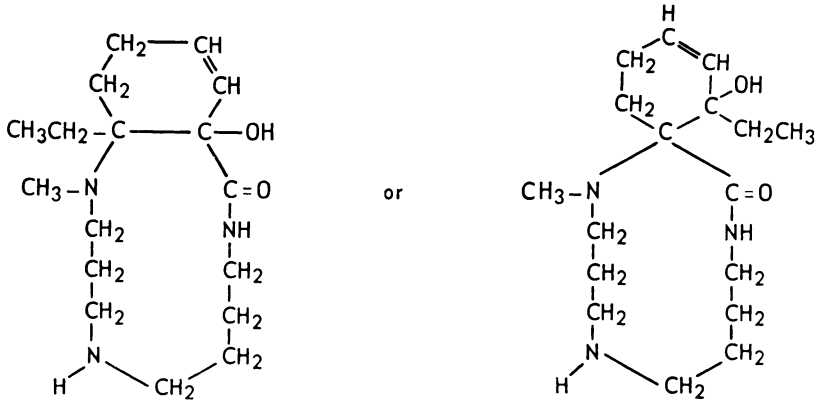


II

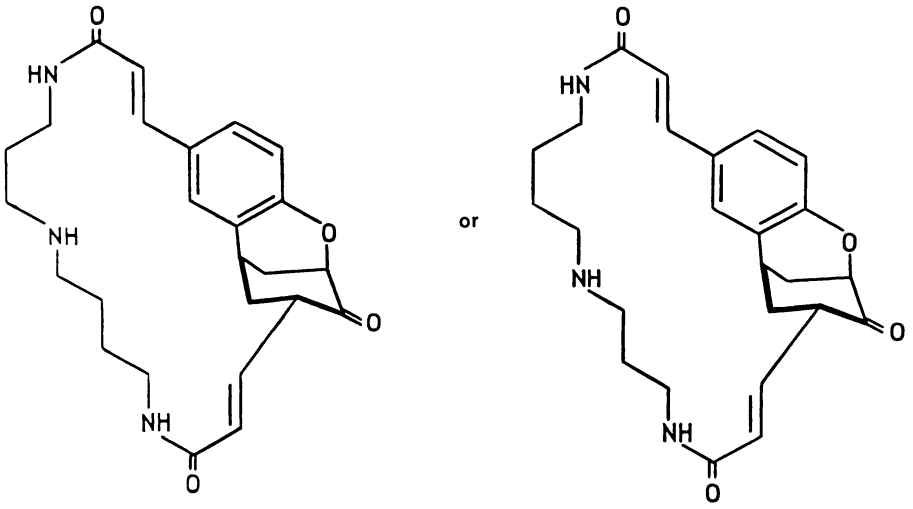
Although at first formula I, by comparison with a synthetic derivative, appeared to be the correct structure for deoxypithecolobine (452), more recent evidence by Wiesner *et al.* (personal communication) indicates that the structure is more likely that represented by II. The exact structure of pithecolobine is unclear, but it is thought that a carbonyl group is present at the position marked with an asterisk instead of a methylene group.

2. *Palustrin* is found in several members of the *Equisetum* genus; in *Equisetum palustre* (horsetail) it is present in 0.01 to 0.02% concentration (dry weight basis), and probably accounts for most of the toxicity of this plant for cattle. In some areas *E. palustre* is widely distributed, and the toxic effects on cattle present a very serious economic problem. The toxic manifestations have been described as irritability, fear, loss of appetite, intestinal disturbances, decrease in milk supply, and a tendency to abnormal births (111).

Although palustrin was isolated in crystalline form in 1936, very little was known of the structure until recently. The following formula is based on extensive analytical and degradative procedures (33, 94, 111, 112), but has not yet been confirmed by synthesis:



3. *Lunarine* has been crystallized from the seeds of *Lunaria biennis* Moench (43, 44, 314, 315); spermidine accounts for all nitrogen in the molecule. The following structures have been proposed for this alkaloid:



E. Intracellular distribution of polyamines

The above sections have been concerned with the overall concentration of polyamines in various tissues. It is very important to know where these amines are located within the cell, but, as yet, few dependable data are available on their intracellular distribution. Obtaining such data is complicated by redistribution of intracellular compounds after disintegration of the cells. The basic polyamines have a high affinity for various acid substances, such as phospholipids (326) and nucleic acids (see section IV), and may be concentrated by those parts of the cell that have a high concentration of these components. Therefore, analyses of cell walls or of various particulate and soluble fractions do not necessarily give any indication of the distribution of the polyamines before the cells were disrupted (28, 477). Indeed, we have found that labeled polyamines added to a liver homogenate are rapidly taken up by the particulate fractions.

This objection does not appear to hold for the reported presence of polyamines in ribosomes of *E. coli*, since Cohen and Lichtenstein (78) showed that the polyamines in these ribosomes do not exchange with the polyamines in the bacterial extract. In their studies 12 to 15% of the polyamines in *E. coli* were in the ribosomal particles; this amount of 1,4-diaminobutane and spermidine is sufficient to neutralize 8% of the RNA-phosphate of the ribosomes. Polyamines have also been described by Spahr (393) and by Zillig *et al.* (477) in ribosomes isolated from *E. coli* and from liver, but secondary redistribution during isolation was not ruled out. In the experiment of Spahr the polyamines accounted for 0.4% of the dry weight of *E. coli* ribosomes and for 75% of the ninhydrin-reactive material in a trichloroacetic acid extract.

The presence of polyamines in a nonexchangeable form in the ribosomal par-

ticles of *E. coli* as described by Cohen and Lichtenstein (78) is particularly interesting and rather surprising, since this would not have been expected from previous work on ribosomal structure. In contrast to the ribosomes of *E. coli*, those from guinea pig pancreas rapidly take up labeled spermine from the medium (378). The only other structures shown to contain polyamines in a nonexchangeable form are the even-numbered T-bacteriophages (section I-C).

Further work on the distribution of the polyamines within the cell would be very helpful in indicating their function. For example, if we knew that a large fraction normally occurs in the nucleus, this would emphasize a polyamine-nucleic acid relationship. On the other hand, if the polyamines were associated with the membrane or wall, this would emphasize a permeability relationship. None of the data obtained so far answers this question adequately since redistribution was not excluded. A novel approach is described in the recent report of Silver (379) in which he showed that polyamines are transferred from an Hfr *E. coli* strain to a recipient strain during bacterial conjugation; unfortunately here also the possibility of secondary uptake of polyamines from the medium invalidates the parts of the experiments that are concerned with polyamine transfer. Special techniques will be necessary to avoid the technical problem posed by redistribution and secondary uptake. Perhaps autoradiography of intact cells after tritium-labeling in tissue or cell culture will be useful.

F. Assay procedures

The following procedures have been used to obtain the data that have been summarized in the past few pages. Unfortunately, as will be indicated below, all available methods have definite limitations, and there is still no simple, specific method for the quantitative determination of the various polyamines. It is usually necessary to carry out one or more preliminary steps to separate the polyamines from each other and from other cellular materials; then the amount of polyamine present is determined by one of the quantitative assays described below.

1. *Isolation* of the relatively insoluble phosphate or picrate salts was the analytical procedure used to obtain the early data on the distribution and concentration of spermine. The procedures, however, were very time-consuming, and considerable losses occurred. Subsequently the more insoluble spermine diflavianate was utilized as the basis of the microcrystallographic procedure of Puranen (35, 114, 135, 316) and of the extensive analytical determinations of Hämäläinen (152) (table 1A). The comparable salts of spermidine and of the diamines are more soluble than those of spermine, and thus are even less suitable for the quantitative estimation of these amines in tissues.

Distillation from an alkaline medium is another purification procedure that has been used to separate the amines from nonvolatile materials. An obvious limitation of this method is the likelihood of hydrolysis of amine derivatives under these vigorous conditions. Further, no recovery experiments have been reported with tissues containing low concentrations of amines.

Extraction of the amines into either n-butyl alcohol or t-butyl alcohol, after the addition of a Na_2SO_4–Na_3PO_4 salt mixture (265, 343), is the most useful pro-

cedure for purification of amines prior to ion-exchange chromatography. The amines are extracted into the butanol layer, while compounds containing acid groups, such as amino acids or protein, remain in the aqueous layer. A disadvantage of this extraction technique for the purification of unknown extracts is that any polyamine derivatives that have acid groups will not be extracted into the butanol layer, and that labile derivatives may be altered during the extraction.

2. *Chromatography and electrophoresis.* The most useful methods for the separation and isolation of polyamines today are based on *ion-exchange chromatography.* The acidic resins (*e.g.*, Dowex 50 or XE-64) retain the very basic polyamines more tightly than most tissue components, resulting in a separation of the polyamines (343, 419). By the appropriate selection of eluting agents, 1,4-diaminobutane, spermidine, and spermine can be separated easily from each other. Usually the amount of amine in each eluate is then determined by the reaction with dinitrofluorobenzene (101, 343).

In terms of sensitivity, these methods are far superior to isolation by crystallization, but they are still rather cumbersome when used for multiple quantitative determinations of amines in numerous tissue samples. Furthermore, during the past few years, we have become aware of the presence of other compounds in biological materials that are not easily separated from these compounds, *e.g.*, monoacetylspermidine; monoacetylspermine; "glutathione" derivatives of spermine and spermidine; homologs of 1,4-diaminobutane, spermidine, and spermine; agmatine; and S-adenosylmethionine, as well as unidentified materials. The presence of these compounds represents a serious limitation on the routine use of the ion-exchange procedures, and in order to increase the specificity of the determinations, it is usually essential to carry out further analytical procedures on the eluates, such as paper electrophoresis or paper chromatography. Very few of the analyses reported in the literature have been obtained with sufficient attention to all of these precautions.

Specific details on the procedures that have been published for ion-exchange chromatography are presented in references 103, 167, 343, 375, 408, 419, 438. References 103, 408, 419, 438 contain diagrams summarizing the elution patterns of the various amines. Each procedure has definite advantages and limitations. Chromatography on *Dowex 50* (H^+*-form*), a sulfonic acid cation-exchanger, using HCl for elution, is particularly useful prior to isolation procedures or radioactivity measurements, since the excess HCl can be easily removed by evaporation. HCl elution, however, suffers from the obvious defect that the material is subjected to strong acid during the elution; although this would not affect the polyamines, it might hydrolyze labile derivatives. Another defect of this chromatographic method is that 1,4-diaminobutane is not adequately separated from its homologs or from ornithine or lysine. A serious fault of this procedure for certain enzymatic experiments is the inadequate separation of S-adenosylmethionine from spermidine and spermine. In most instances this is not significant, but it represents an important problem in biosynthetic experiments with C^{14}-methionine, since S-adenosylmethionine-C^{14} may be formed. Thus, in some of the pub-

lished experiments on the incorporation of C^{14}-methionine into polyamines, the label may have been associated with accumulated S-adenosylmethionine rather than with newly synthesized polyamines.

Adsorption on a column of *Amberlite IRC-50 (XE-64 K^+-form)*, a carboxylic acid cation-exchanger, followed by gradient elution with a buffered sodium sulfate–sodium phosphate mixture, affords the best separation of the amines from the amino acids. However, it still does not afford sufficient separation of the various diamines from each other. This procedure has the marked disadvantage that the eluates contain high concentrations of salt. For some purposes chromatographic separation on *Amberlite IRC-50 (H^+-form)* can be used. With this resin the polyamines are easily eluted with relatively weak acetic acid. This method is particularly useful for the separation of small amounts of C^{14}-spermidine from C^{14}-S-adenosylmethionine. Chromatography on IRC-50 in the H^+-form, however, is very sensitive to the salt concentration of the original solution.

Paper chromatography has been used extensively for identification of the polyamines. Solvents and R_f values for spermidine and spermine may be found in references 2, 3, 30, 50, 57, 103, 111, 120, 122, 165, 212, 320, 341, 375, 376, 378, 441, 469, 470. R_f values for the diamines may be found in references 30, 50, 57, 81, 103, 111, 122, 165, 262, 295, 301, 324, 341, 375, 378, 388, 440, 441, 470. Paper chromatography of the dinitrophenyl derivatives of the diamines has also been described (21, 477). Details on procedures for *paper electrophoresis* are given in references 50, 122, 157, 165, 195, 250, 262, 320, 375, 378, 440, 441, 470, 477. In these procedures, the amines are usually detected with a ninhydrin spray. *Thin-layer chromatography* has been utilized to separate spermine and spermidine (320, 375).

Gas chromatography seems particularly suitable for polyamine assays, and several preliminary methods have been developed (65, 74, 195, 384, 399). As yet, however, none of the systems is suitable for the quantitative separation and assay of the various amines in tissue extracts.

3. *Quantitative and qualitative assays.* Several *color reactions* have been used for the detection and the assay of the polyamines. In general, these are not sufficiently specific to be used without prior purification procedures. The most common reactions are those of *dinitrofluorobenzene* and *ninhydrin* mentioned above. Differences in the spectra of the derivatives obtained in the dinitrofluorobenzene reaction have been used to distinguish between primary and secondary amines (101, 264). *Copper carbonate* gives a blue color (Tokuoka reaction) with spermine, and, to a lesser extent, with spermidine, and has been widely used for various clinical determinations (18, 79, 89, 118, 119, 129, 144a, 294, 298, 312, 332, 333, 376, 428, 429, 430, 472). Until the specificity and sensitivity of this method are further evaluated, however, it is difficult to interpret the data. Another colorimetric method involves the coupling of the amines with *diazotized 4-nitroaniline* (250). The *Dragendorf* reagent has been used as a spray to detect spermidine and spermine (2), and *amido black* as a sensitive reagent for the quantitative determination of spermidine and spermine (320). Spermine has been detected also by a *platinic iodide* spray (290).

Bioassay procedures for the polyamines have been introduced by Herbst *et al.* (164–168), using *Hemophilus parainfluenzae* 7901 as the assay organism. This organism requires 1,3-diaminopropane, 1,4-diaminobutane, agmatine, spermidine, or spermine for growth. Since each of these amines causes a growth response, this assay alone cannot distinguish one of these amines from another. Therefore, bioassay has usually been combined with paper chromatography or paper electrophoresis to effect preliminary separation. *Aspergillus nidulans* pu_1 can also be used for bioassay purposes since it requires 1,4-diaminobutane for growth (390).

A specific, sensitive *enzymatic method for spermidine* has recently been developed in this laboratory by Bachrach and Oser (29). Spermidine is converted by dried *Serratia marcescens* cells to 1,3-diaminopropane and Δ^1-pyrroline; the latter reacts with *o*-aminobenzaldehyde to produce a yellow color. Other amines either are not oxidized by the enzyme or do not form Δ^1-pyrroline (see section X-B). A combined *enzymatic-fluorometric* assay for spermine and spermidine has recently been introduced by Unemoto *et al.* (438). The amines are oxidized by beef plasma amine oxidase (see section X-A), and the products are converted to fluorescent materials by reaction with resorcinol.

4. *Measurements of radioactivity.* The availability of C^{14}- and H^3-labeled polyamines (see section XI for synthetic procedures) has markedly facilitated the assay of these compounds. Very small amounts can be determined if compounds of high specific activity are used. The radioactivity of C^{14} and H^3 can be measured by conventional procedures. Unfortunately, however, the polyamines adsorb very actively to materials like glass, Millipore membranes, and cellophane dialysis tubing. Consequently, unless adequate precautions are taken, substantial losses of the polyamines may occur by such adsorption (399). This problem occurs to a greater extent with spermine than with the diamines and triamines, and is most significant if only tracer amounts of the polyamine are used.

II. EFFECTS OF POLYAMINES AT THE CELLULAR LEVEL

Polyamines as microbial growth factors. The earliest definitive demonstration that these amines can have an essential biological role was the finding that they are required for the growth of certain microorganisms. The first of these studies were those of Herbst *et al.* (164, 166, 167) on *H. parainfluenzae* 7901. In the absence of added polyamine no growth occurs on a purified medium. Growth is obtained upon addition of small amounts of 1,3-diaminopropane, 1,4-diaminobutane, or any one of about twenty other synthetic derivatives of these diamines, including spermidine and spermine; 1,5-diaminopentane is inactive.

Sneath (390) described a mutant (pu_1) of *A. nidulans* that has an absolute requirement for 1,4-diaminobutane. Several other compounds, including spermine, ornithine, arginine, lysine, 1,3-diaminopropane, and 1,5-diaminopentane, are ineffective, and only a small growth response is found with spermidine. The occurrence of a mutant with this specific growth requirement for 1,4-diaminobutane indicates that 1,4-diaminobutane or a derivative has an essential function in the cell. The absence of such mutants in *E. coli* does not exclude an essential

function for polyamines in *E. coli* since there are several other possible explanations for the lack of mutants. Some possibilities follow: (a) in the absence of polyamines, other compounds, such as divalent cations or basic proteins, can substitute for them; (b) two pathways may exist for the synthesis of 1,4-diaminobutane, *i.e.*, directly from ornithine or from arginine *via* agmatine (section VIII); both pathways would have to be blocked before a requirement is noted, and this is very unlikely; and (c) polyamine-requiring mutations may be "lethal," *i.e.*, these mutants may not be able to survive because of changes in permeability or in DNA structure (section IV), and thus they could not be isolated by the penicillin selection techniques used.

Besides the absolute growth requirements for polyamines shown by *H. influenzae* and *A. nidulans* pu_1, a growth-stimulating effect of polyamines is observed with several other organisms [*Neisseria perflava*, *Pasteurella tularensis*, and *Lactobacillus casei* (127, 207, 266, 270, 284, 432)]. In most of these cases, however, the growth-stimulating effect is probably indirect and is presumably due to the ability of polyamines to prevent lysis of various fragile bacteria in media of low ionic strength [*e.g.*, *P. tularensis* (266, 268), *Achromobacter fischeri* (267), *S. aureus* (326)].

Stabilizing action of polyamines on bacterial spheroplasts and protoplasts.[5] The discovery of the stabilization of osmotically fragile bacteria by small amounts of spermine indicated that in some unknown way spermine affects the strength of the bacterial cell wall or membrane. This led to the demonstration by Mager (268) and C. W. Tabor (400, 402) of comparable protection of bacterial spheroplasts and protoplasts. When *E. coli* are treated with lysozyme and ethylenediaminetetraacetate in a 20% sucrose medium, the bacteria are converted to spheres (spheroplasts) that lyse if the osmotic pressure is lowered (329). Protection against lysis is afforded by 0.001 M spermine, spermidine, $CaCl_2$, quinacrine, or streptomycin; comparable concentrations of KCl, NH_4Cl, $MgCl_2$, ornithine, lysine, or 1,4-diaminobutane are inactive. Spermine has a protective effect also on spheroplasts of *E. coli* produced by penicillin treatment and protoplasts of *Micrococcus lysodeikticus* made by treatment with lysozyme (146, 268, 402).

The striking aspect of these results is the low concentration of spermine (0.001 M) needed to prevent lysis, compared to the 0.5 M sucrose usually used. The interior of the cell has a higher osmotic pressure than the external medium; the cell is prevented from swelling even in distilled water by the rigidity of its cell wall. When the wall is damaged by lysozyme or its synthesis is inhibited by penicillin this restraint is absent and the spheroplast swells until its membrane ruptures, *i.e.*, lysis occurs. The 0.5 M sucrose prevents lysis by raising the osmotic

[5] The term protoplast refers to those bacterial structures formed by complete removal of the cell wall components by the action of lysozyme (as with *M. lysodeikticus*). When osmotically unstable structures are formed by disruption of certain linkages without complete removal of cell wall (as by the action of penicillin or by the action of lysozyme-EDTA on gram-negative bacteria), the term spheroplast is used. Sometimes, however, the term protoplast is used in the literature for any unstable structure formed by the action of lysozyme.

pressure of the environment to that of the contents of the spheroplast. Spermine in a concentration of 0.001 M does not increase the osmotic pressure significantly and therefore presumably acts by strengthening the spheroplast membrane.

To account for stabilization of this kind by spermine and the other compounds, it seems reasonable to assume that each of these substances, being basic, forms complexes with acid groups in the cell membrane and so reduces a repulsive effect of these groups without diminishing the cohesive factors, such as hydrogen bonding and van der Waals' forces. This postulation is supported by the observations that spermine does indeed bind to spheroplasts, phospholipids, and cell-wall components (28, 326, 402). This is very similar to the binding of Ca^{++} to membranes, fatty acids, and phospholipids, and to the well-known stabilizing action of Ca^{++} in many systems.

Miscellaneous effects. Another indication of an interaction between polyamines and acidic groups on the cell-surface is the *aggregation* that is observed when sufficient spermine is added to suspensions of gram-positive bacteria (326), gram-negative bacteria in the rough form (326), or *E. coli* spheroplasts (402).

Spermine partially inhibits the delayed hemolysis observed when *reticulocytes* are suspended in saline (263). 1,4-Diaminobutane and other diamines prevent hemolysis of sensitized sheep erythrocytes possessing the first component (C'I) of hemolytic complement; this is due to the release of the C'I from the cells by the diamine (455).

Studies on the effect of spermine on the motility of *spermatozoa* have shown an increase in the vibrational motility (407), but no effect on the duration of motility (155, 327). Spermine decreases the deleterious effects of dilution on rabbit spermatozoa, but not on bull, ram, or human spermatozoa (449).

The polyamines have been shown to act as *growth factors for a Chinese hamster cell line in culture* (151). These studies have permitted the development, for the first time, of a completely defined protein-free medium, using spermine (10^{-6} M), spermidine, or 1,4-diaminobutane instead of fetuin, and linoleic acid instead of serum albumin (151). Good clonal growth is obtained, although the plating efficiency is low.

Agmatine decreases the division time of *embryonic neuroblasts* (grasshopper) (348).

III. EFFECTS OF POLYAMINES ON SUBCELLULAR PARTICLES

Ribosomes. Several laboratories have reported that larger ribosomal particles are obtained from *E. coli* (78, 80) and *S. typhimurium* (283) in the presence of added polyamines. This effect is observed either when spermine is added to the growth medium or when spermidine is present in the suspending fluid used for the preparation of the ribosomes. A similar result can be obtained if Mg^{++} is added to the suspending fluids, but the greatest effect is found in the presence of both Mg^{++} and the polyamine. Since 15% of the polyamines of an *E. coli* extract are in the ribosomal fraction (see section I-E), it was suggested by Cohen and Lichtenstein (78) that "Mg^{++} and spermidine are the naturally occurring clasps on the complex ribosomal components existing in the bacterium." In the absence

of Mg^{++} and spermidine, the larger ribosomal particles dissociate into smaller subunits. Some evidence for stabilization of pancreatic ribosomal particles by spermine has been presented by Siekevitz and Palade (378).

In view of these marked effects of polyamines on ribosomes, it is not surprising that the polyamines affect the activity of systems that incorporate amino acids *in vitro* (171, 172, 283, 305, 427). Nathans and Lipmann (305) studied the transfer of C^{14}-aminoacyl-s-RNA to ribosomes of *E. coli*. A high concentration of Mg^{++} is required for optimal activity, but this can be replaced, at least partially, by spermidine. In the absence of both, the rate is only about 6 % of the optimal rate. Martin and Ames (283) subsequently reported a comparable ability of spermine to substitute for Mg^{++} in the incorporation of C^{14}-phenylalanine into ribosomal protein in a *S. typhimurium* system. To obtain this effect of polyamines, polyuridylic acid, as added "messenger," must be present. In this case, the ribosomes become the limiting component of the system, and the ability of the polyamines to maintain the functional integrity of the ribosomes (100S rather than 70S particles) would account for the increased amino acid incorporation. Spermine can also partially replace the Mg^{++} requirement of a mammalian amino acid incorporating system (171, 172). In both the *S. typhimurium* and the mammalian system, a stimulatory effect of spermine is found only in the presence of suboptimal concentrations of Mg^{++}. Spermidine, agmatine, and 1,4-diaminobutane are also effective, but higher concentrations are necessary.

Another series of observations that indirectly demonstrates the effect of polyamines on subcellular particles is the finding that 80 to 90 % of the ribosomal amylase, ribonuclease, and chymotrypsinogen are released when pancreatic ribosomes are suspended in solutions containing spermine (377, 378). All ribosomal proteins are not released, however, by spermine (378); indeed, in experiments with reticulocyte ribosomes spermine actually inhibits the release of newly synthesized protein from the ribosomes (299). Somewhat related to the latter findings are the observations of Adams and Newberry (5) that the solubilization of D-allohydroxyproline oxidase from particles of *Pseudomonas striata* which is effected by ethylenediaminetetraacetate can be prevented by spermidine, spermine, or Mg^{++}.

Under certain conditions spermidine or spermine can cause the visible aggregation of ribosomes (378, 479) and microsomes (400). The ability of spermine to precipitate ribosomes is the basis of a simplified procedure for the purification of *E. coli* ribosomes (479), since in the presence of the polyamine high-speed centrifugation is unnecessary.

Isolated mitochondria have a slow rate of spontaneous swelling in isotonic media; this rate of swelling can be markedly increased by a variety of "swelling agents," such as succinate or oleate (187, 256). Rapid swelling is also observed if the mitochondria are suspended in hypotonic media (261). Swelling induced by these agents is markedly reduced by either spermine or spermidine at 3×10^{-4} M, or by higher concentrations of 1,4-diaminobutane and 1,5-diaminopentane (169, 400). In our experiments spermine did not affect the swelling induced by phosphate, ascorbate, or thyroxine. The following suggestions may explain the protection of mitochondria by polyamines.

1) The polyamines may bind the phospholipids on the mitochondrial surface because of the strong affinity of the basic amines for this type of compound. Neutralization of these negative charges, which normally repel each other, may make the membrane more impermeable or mechanically stronger.

2) We have found that polyamines markedly decrease the action of the mitochondrial swelling factor ("U" factor), that has been isolated from mitochondria by Lehninger and Remmert (256), and that has been postulated as an agent responsible for the swelling. Since this "U" factor has the characteristics of a long-chain unsaturated fatty acid, these findings are consistent with the ability of polyamines to inhibit mitochondrial swelling due to oleate. In discussing the mechanism of action of the "U" factor, one may speculate that unsaturated fatty acids must be oxidized to induce swelling. We have shown that under certain conditions the oxidation of unsaturated fatty acids (either hematin-catalyzed or auto-oxidation) is inhibited by spermine (401). Consistent with this concept is the report that mitochondrial swelling induced by a variety of agents is prevented by strict anaerobiosis and respiratory inhibitors (187).

Nuclei. Incubation of the lateral roots of beans (*Vicia faba*) with 1,4-diaminobutane, 1,5-diaminopentane, or spermine (5×10^{-4} M) causes inhibition of chromosomal uncoiling in anaphase and telophase, and fusion of metaphase chromosomes into a single mass; these effects on the normal processes of chromosome coiling and uncoiling may be due to condensation of the long strands of nucleoprotein brought about by the polyamine (88). In *V. faba* chromosomal breakage does not occur (88, 331), although both chromosomal aberrations and breakage have been found in *Oenothera* cells during meiosis after 1,4-diaminobutane (281).

The effect of polyamines on isolated rat *liver nuclei* is dependent on their concentration; 0.001 M spermine or spermidine or 0.025 M 1,3-diaminopropane, 1,4-diaminobutane, or 1,5-diaminopentane causes shrinkage of the nuclei with bleb formation and condensation of the nucleoli (17). Higher concentrations cause swelling of the nuclei. Indirect evidence for stabilization of *thymus nuclei* and of nuclear ribonucleoprotein is afforded by the recent note of Amoz and Mager (13) on the stimulatory effect of spermine on the incorporation of C^{14}-leucine into protein in thymus nuclei.

IV. POLYAMINES AND NUCLEIC ACIDS

The most interesting developments in the polyamine area in recent years have been the various studies concerned with the association of these amines with nucleic acids. The polyamines bind tightly to nucleic acids, forming complexes which, if sufficiently concentrated, produce precipitates. These complexes seem to involve a noncovalent linkage between the basic groups of the polyamines and the highly acidic phosphate groups of the nucleic acid. This results in neutralization of the negatively charged phosphate groups that usually repel each other, and therefore in an increase in the net strength of various cohesive forces, such as van der Waals' forces and hydrogen bonding. The resultant increase in the strength of secondary structures, such as the double helix of DNA, may explain many of the stabilizing effects described below.

A. Association of polyamines and nucleic acids

Bacteriophages. Evidence for a close association of polyamines and nucleic acids *in vivo* is the presence of these amines in the T-even series of *E. coli* bacteriophages (section I-C). Polyamines in these bacteriophages do not exchange with polyamines in the medium, and are injected together with phage DNA into bacteria during infection (10, 11, 170). The nonexchangeability of the phage polyamines is presumably due to the impermeability of the protein coat of the T-even bacteriophages since there is no evidence for any covalent bonds between the polyamines and the phage nucleic acids.

Precipitation of nucleic acids and polynucleotides. Prior to the bacteriophage studies, there was already considerable information on the formation of polyamine-nucleic acid complexes *in vitro.* In 1948, Bichowsky-Slomnitzki (39) showed that nucleic acids can inhibit the antibacterial action of spermine and spermidine. Subsequently, Razin and Rozansky (326) showed that spermine and other polyamines (but not diamines) precipitate nucleic acids, and that the amines are found in the precipitate. More recently, spermine has also been shown to precipitate the ribonucleic acids of tobacco mosaic (34) and turnip yellow mosaic viruses (289), s-RNA (67), and various polynucleotide polymers (185). Spermine also forms complexes with nucleic acids which are insoluble in 66% ethanol (203, 204, 470).

Precipitation of nucleic acids and polynucleotide polymers by polyamines depends on ionic strength (34, 326, 416), pH, and the nucleotide composition (67, 185). Fractional precipitation by spermine can be used to separate mixtures of polyribonucleotide copolymers (115, 185) or to effect a partial separation of RNA fractions (67, 77). The mechanism of the precipitation of nucleic acids by spermine is not known. Although precipitation may indicate aggregation resulting from spermine bridges between nucleic acid molecules, neutralization of the negative phosphate groups by spermine would favor aggregation even without bridges, since van der Waals' forces would no longer be opposed by the repulsive forces between phosphate groups.

Conductimetric and spectrophotometric studies. There are definite limitations in the use of precipitation techniques for the study of complex formation, since precipitation of a complex is dependent on its solubility as well as on its formation. More useful techniques are those in which dilute solutions are used, and in which precipitation does not occur. An example is the study of Felsenfeld and Huang (115, 117) on the effect of different concentrations of spermine on the conductivity of dilute solutions of DNA and s-RNA, demonstrating a tight spermine-nucleic acid binding (one equivalent of spermine per mole of phosphate). They also used spectrophotometric techniques to show that polyamines facilitate the formation of two-stranded copolymers from single-stranded polynucleotides (115, 116). These techniques depend on the lower optical densities ("hypochromic effect") observed with a helical copolymer, as compared to the optical densities of the separate constituent chains. For example, when diamines are added to mixtures of polyadenylic acid (poly A) and polyuridylic acid (poly

U), the optical density decreases, presumably resulting from the formation of the two-stranded poly (A + U). Similarly, two-stranded polyinosinic-polycytidylic acid can be formed from polyinosinic and polycytidylic acids. These results presumably reflect the binding of the amines to the polynucleotide; when the phosphate groups are neutralized by complex formation, it is easier for two-stranded copolymers to form.

Technical problems of adsorption and precipitation. Although the studies described above demonstrated the binding of various polyamines to nucleic acids, no extensive study of the dissociation constants of the complexes of polyamines with various nucleic acids has been reported. We have attempted to obtain such data in our laboratory by equilibrium dialysis. Despite direct evidence by this method for polyamine-DNA binding, we have not yet obtained dependable equilibrium constants because of several technical problems that must be overcome in all experiments involving spermine and DNA. If the concentrations of spermine and DNA exceed very low levels, gross aggregation and precipitation occur and this interferes with dialysis studies, optical density measurements, *etc.* This critical concentration depends on the ionic strength. For example (416), in 0.03 M NaCl at pH 6.2 turbidity occurs in a solution containing 3×10^{-5} M spermine and 15 μg of DNA per ml; higher concentrations can be tolerated at higher ionic strengths. On the other hand, if, in the presence of low ionic strengths, the spermine concentration is kept low in order to avoid precipitation, the data are complicated by adsorption of the spermine to dialysis tubing and glass (section I-F, 4).

Another technical problem related to the high affinity of DNA for polyamines is the presence of polyamines in some preparations of DNA. In the water–phenol system that is commonly used for the preparation of DNA, the polyamines stay in the aqueous layer with the DNA (10, 374). DNA may be freed of polyamines by successive precipitation of DNA by ethanol from solutions of high ionic strength.

B. Heat denaturation of nucleic acids

Deoxyribonucleic acids. Much of the evidence for DNA-polyamine interactions is based on the ability of these polyamines to decrease the denaturation of DNA that is caused by heating. The denaturation presumably results from disruption of the helical DNA structure; this structure appears to be markedly stabilized by the presence of polyamines. Both the denaturing effect of heat and the protective action of the amines can be measured either biologically or spectrophotometrically.

The biological assay involves heating transforming-DNA obtained from *Bacillus subtilis*, and measuring the transforming ability that remains. In the presence of polyamines, considerably higher temperatures are necessary to inactivate the DNA. For example, in our experiments (415) the transforming ability is lost at 70 to 75°; in the presence of 10^{-4} M spermine heating to 90° is necessary. As little as 2×10^{-6} M spermine has some protective effect. Spermidine is effective, but a higher concentration (10^{-4} M) is required; only a small effect

is observed with 1,4-diaminobutane or 1,5-diaminopentane, even at a concentration of 10^{-2} M.

The spectrophotometric methods are more convenient, and depend on the increase in optical density at 260 mμ that occurs when DNA is denatured ("melted") by heating; the "melting-temperature" (T_m) is defined (98) as the temperature at which one-half of the maximum increase in optical density occurs. The effect of polyamines in increasing the "melting-temperature" is most clearly demonstrated in solutions of relatively low ionic strength (416). For example, in 0.03 M NaCl the "melting-temperature" of calf thymus DNA is 76.1°. This "melting-temperature" is increased 2° by as little as 1×10^{-6} M spermine, and 9° by 1.7×10^{-5} M spermine. No effect is observed with comparable concentrations of spermidine, 1,4-diaminobutane or $MgCl_2$; at higher concentrations (0.0001 M to 0.001 M), however, these compounds also increase the "melting-temperature." These effects are not due to changes in the ionic strength, since even higher concentrations of NaCl (0.1 to 0.2 M) are required for comparable increases in the "melting-temperature."

When the effect of added polyamines is studied at a higher ionic strength, protective effects are still observed; however, the increases in the "melting-temperature" due to the polyamine are less striking, and higher concentrations are required. Although the protective effect of spermine and the diamines is still present at the higher ionic strength, $MgCl_2$ no longer has such an effect (274, 416). In a study of the relative activity of a homologous series of diamines, the protective effect is optimal with 1,5-diaminopentane (Mahler *et al.*, 271–274). The protective action of the various polyamines against heat denaturation of DNA is consistent with the concept that these amines form complexes with the phosphate groups and thus stabilize the helix by diminishing the repulsive forces between these negatively charged groups. Another stabilizing factor could be spermine bridges between two DNA chains, but no evidence for such linkages has been reported. The differences among the various amines in their stabilizing effects are probably directly related to their affinities for DNA.

Mahler and Mehrotra (273) have suggested an additional complex of the polyamines with the adenine or thymine components of the DNA, since the increase in "melting-temperature" due to the addition of spermine or diamines varies with the base composition of the DNA (273, 275). At a given concentration of polyamine, the absolute increase in the "melting-temperature" is highest in those DNA samples with the highest percentage of adenine plus thymine (A + T), and relatively low in those samples with a high content of guanosine plus cytosine (G + C). However, other explanations for these observations have not been ruled out. The DNA samples with a high $\frac{A + T}{G + C}$ ratio have a much lower "melting-temperature" than samples with a low $\frac{A + T}{G + C}$ ratio. Hence, the experiments with the different types of DNA are necessarily carried out in different ranges of temperature. It is reasonable to anticipate that this difference in temperature will affect such factors as the amount of amine bound to DNA at a given concentration of amine, the dissociation constants of the phosphate groups,

and the strength of the various cohesive and repulsive forces within the DNA molecule; it is necessary to exclude these factors before a specific nucleoside-amine binding can be accepted. One argument against such a complex is the lack of any significant spectral shift (273) when polyamines are added to a DNA solution; however, it is not certain that such a shift would necessarily occur. Another reason to question a specific interaction of the polyamines with the nucleoside components is that similar results are obtained with Mg^{++}: the effect of Mg^{++} in increasing the T_m is greater in DNA samples that have the highest $\frac{A + T}{G + C}$ ratio, and no spectral shift is observed (99). On the other hand, if these arguments were valid, the changes in T_m due to changes in ionic strength should show a comparable dependence on the $\frac{A + T}{G + C}$ ratio. Dove and Davidson (99) have shown, however, that this is not the case: the increase in T_m that results from an increase in ionic strength caused by the addition of sodium perchlorate is not affected by the $\frac{A + T}{G + C}$ ratio.

Ribonucleic acid. Mitra and Kaesberg (289) showed that spermine and bis-(3-aminopropyl)-amine markedly increase the "melting point" of RNA from turnip yellow mosaic virus. On the other hand, Mahler and Mehrotra (273) found no effect of 1,5-diaminopentane on the thermal denaturation of tobacco mosaic virus RNA or of s-RNA preparations. With polyadenylic acid in acetate buffer ("acid poly A") the diamines surprisingly *decrease* the denaturation temperature; this effect was interpreted as indicating labilization of the helical structure (273).

C. Inhibition of DNA shearing by spermine

Spermine (10^{-5} M) prevents the shearing of DNA that results from stirring dilute solutions, presumably by forming a spermine-DNA complex (197). In contrast to the heat denaturation experiments (see above), no protective effect is observed with spermidine, 1,4-diaminobutane, $MgCl_2$, or NH_4Cl. Therefore, it seems likely that the spermine complexes with the DNA in a linear fashion along the chain, thereby introducing additional C—C bonds that have to be sheared by the hydrodynamic forces. Although cross-linkage between DNA molecules with spermine bridges is also a possibility, this is rendered somewhat unlikely by the observation that the protective effect of spermine is independent of the concentration of DNA.

D. Effect of polyamines on enzymatic reactions involving nucleic acid substrates

The mechanism of the polyamine effects on the enzyme systems listed below is still not known, but, for the most part, the effects seem to be due to actions of the polyamines on the nucleic acid substrates, rather than on the enzymes themselves. If the nucleic acid were removed from the solution by precipitation by the polyamines, or if polyamine-nucleic acid complexes were formed, this would alter the effective concentration of nucleic acid in any reaction in which it is a substrate, a primer, or an inhibitory product. These possibilities may also be

significant *in vivo*, and the polyamines may be important in controlling nucleic acid turnover.

Nucleases. Although the polyamines inhibit the degradation of RNA and DNA by whole cells and by various tissue preparations (12, 134, 163, 204), this need not represent a specific effect on nucleases. In addition to binding of the nucleic acid substrates, the amines may be acting indirectly by stabilizing the cells or ribosomes (sections II and III), and thereby diminishing the release of "latent" nucleases. With purified ribonuclease (RNase) and deoxyribonuclease (DNase), the spermine effect has ranged from slight activation to inhibition (12, 204, 289, 378). Keister (204), for example, showed that spermine inhibits RNase at pH 7, but stimulates at pH 5. On the basis of kinetic studies he considered that spermine inhibits the RNase reaction by binding the substrate. In any crude incubation mixtures these effects of spermine on the nuclease reactions would affect the concentration of nucleic acids present, and thereby might indirectly affect the activity of other systems (see below) that use nucleic acids as substrates.

RNA polymerases. Different effects of spermine on RNA polymerase have been reported from several laboratories, using different polymerase preparations; it is not possible to present, at this time, a single explanation of the various findings. Spiegelman (394) found that spermine appears to prolong the action of the RNA polymerase of *E. coli* membrane fractions. This effect was confirmed by Doerfler *et al.* (96), using a crude *E. coli* system, although they found that larger amounts of spermine are inhibitory. Using a purified *A. vinelandii* polymerase, Krakow showed that spermine causes a 2- to 3-fold stimulation if native DNA is used as a primer (243). With heat-denatured DNA, on the other hand, spermine has no effect, while with synthetic polyribonucleotides as the primer, spermine inhibits the polymerase. Similar effects of the polyamines have also been reported by Fox *et al.* (129a, b) for a purified *M. lysodeikticus* RNA polymerase. With a crude chick embryo polyribonucleotide polymerase, low concentrations of spermidine or 1,5-diaminopentane inhibit the reaction, while high concentrations stimulate moderately (73).

Polynucleotide phosphorylase. Spermine and spermidine have no effect on a partially purified polynucleotide phosphorylase from *Clostridium perfringens*, even though this enzyme has an absolute requirement for long-chain polybasic substances (97).

α-Glucosyl transferases. Spermine (1×10^{-4} M) stimulates T2- and T6-glucosyl transferases, enzymes that glucosylate the hydroxymethylcytosine of DNA (478). Spermidine, 1,4-diaminobutane, and 1,5-diaminopentane are relatively inactive; 0.01 M ammonium sulfate, however, is as effective as 0.0001 M spermine. The secondary structure of DNA is very important for this reaction, and it is possible that spermine may stimulate the reaction by stabilizing this structure.

Aminoacyl-s-RNA synthetase. The main effect of polyamines in stimulating protein biosynthesis *in vitro* (section III) is related to stabilization of the 100S ribosomes rather than to the formation of aminoacyl-s-RNA. However, some effect of spermine on an *E. coli* aminoacyl-s-RNA synthetase has been reported by Doctor and Mudd (95), but only if liver or yeast s-RNA is used as an ac-

ceptor; no effect of spermine is found if *E. coli* s-RNA is the acceptor. Perhaps spermine protects liver or yeast s-RNA from contaminating nucleases.

"Latent" RNase. 1,4-Diaminobutane (0.5 M) inhibits the activation of a "latent" ribosomal RNase that occurs in the presence of urea (477); the most likely explanation of this effect is stabilization of the ribosome particles.

E. Miscellaneous

Bacterial and mammalian transformation. In section IV-B we discussed our experiments on the stabilization of the transforming-DNA of *B. subtilis* against heat denaturation. In other experiments with unheated *B. subtilis* DNA, we have found that the addition of spermine to the transforming mixture results in a 3-fold increase in the number of transformants (415).

A striking effect of spermine on transformation of mammalian cells in culture has been reported by Szybalska and Szybalski (398); transformants are obtained only in the presence of spermine. No other amines were tested. Further developments in this area will be of particular importance since this report represents the first claim for unequivocal transformation of mammalian cells *in vitro*.

In both cases it is possible that the observed effects are due to protection of the DNA against nucleases or against other destructive influences. However, an effect of the polyamine on the competence of the recipient cells cannot be ruled out.

Infective tobacco mosaic virus nucleic acid is 95 % inactivated by 0.001 M spermine. This inactivation is partially prevented by 0.3 M sodium acetate or sodium chloride, and is almost completely abolished by 0.02 M sodium citrate (34). Although histones and protamines enhance the plaque-forming capacity of polio virus RNA, spermine, spermidine, and 1,5-diaminopentane have no effect (389).

F. Discussion

Although several lines of investigation have elucidated definite spermine-nucleic acid interactions *in vitro*, the role of the polyamines *in vivo* is still unclear. Unfortunately we have very little information on which to base any definitive conclusions about the significance of the polyamines *in vivo*. As we have mentioned above, one of the most serious gaps in our knowledge is our present inability to localize the amines within the cell, and to know, in particular, whether they are present in higher concentrations within the nucleus.

Very closely related to any discussion of the relationship between polyamines and nucleic acids *in vivo* is the structural and functional relation of the basic proteins (protamines and histones) to nucleic acids. Despite considerable interest in this aspect of the problem over many years there are still too few data to support definitive conclusions (for references and discussion see 15–17, 72). Many of the effects of polyamines that have been reviewed above are similar to those observed with histones, protamines, and polylysine. These compounds, for example, also precipitate nucleic acid (72, 326) and protect DNA against heat denaturation (184, 416). The roles of Mg^{++} and Ca^{++} also have to be considered, since these cations have comparable effects in many instances. It is noteworthy, how-

ever, that much higher concentrations of these divalent cations have to be used to accomplish the same effect as much lower amounts of polyamines or histones.

Another consideration in discussing the relationship of polyamines or histones to nucleic acids is their effect on the physical state of the nucleic acids within the cell or bacteriophage, since, as we have already emphasized, DNA is easily precipitated by low concentrations of polyamines or histones. For example, it is likely that the polyamine-DNA complex within the T-even bacteriophages is insoluble, since both components are sufficiently concentrated to cause precipitation. Similarly, if the amines known to be present in the tissues were concentrated in the nuclei, one would also expect precipitation, although the occurrence of precipitation would be affected by the presence of other proteins and of inorganic salts.

V. POLYAMINES AND BACTERIOPHAGES

In sections I and IV we discussed the presence of polyamines in certain bacteriophages of *E. coli*. In this section we will consider various other studies on bacteriophages, including, in particular, the effect of polyamines on their viability and replication.

Stabilizing effects. A striking effect of the polyamines is their ability to protect certain bacteriophage suspensions against various forms of inactivation. The destructive effect of heat, dilution, or freezing-thawing on π (a urea-shocked bacteriophage that infects protoplasts) is markedly decreased by diamines (0.01 M) and by spermidine (131). At a given concentration the most effective diamine is 1,5-diaminopentane. Similarly, the inactivating effect of citrate or ethylenediaminetetraacetate on *E. coli* bacteriophage T5 (presumably caused by binding divalent cations) is markedly diminished by either spermidine or spermine, even when the amine concentration is as low as 10^{-7} M (414). 1,4-Diaminobutane and 1,5-diaminopentane are also effective, but higher concentrations (5×10^{-5} M) are required. The extremely low effective concentrations presumably indicate that the polyamines have a high affinity for the bacteriophage DNA. Fraser and Mahler (131) pointed out that the N—N distance in 1,5-diaminopentane (7.30 Å) is similar to the distance between the phosphate oxygen atoms in DNA (7.65 Å).

Effect of changes in polyamine content on bacteriophage viability and replication. The polyamine content of the T-even bacteriophages can be altered by growing the host *E. coli* in media containing spermidine or spermine. Despite large changes in the total and relative concentrations of each of the polyamines within the isolated bacteriophages, there is no effect on the infectivity, burst size, or the sensitivity to ultraviolet irradiation (10).

It has not been possible to produce T-even bacteriophages without *any* polyamines since *E. coli* always contains polyamines, and these are transferred to the bacteriophages. Kim (208) tried to produce *E. coli* without polyamines by selecting an *E. coli* strain that can easily adapt to growth on 1,4-diaminobutane, and that has an active enzyme system for the metabolism of this amine (section X-B). However, although the 1,4-diaminobutane level can be markedly lowered in

these bacteria by nitrogen starvation, significant amounts of spermidine and acetylated spermidine remain. Despite the low 1,4-diaminobutane level, bacteriophages infect these bacteria normally, and a normal burst size results.

Added spermine has very little effect on the viability of either T-even or T-odd bacteriophage suspensions (31, 296). Kay has reported (201), however, that the addition of spermine (1×10^{-4} M) to *E. coli* 518, infected with bacteriophage 3, inhibits the burst. Similarly, the burst is inhibited when *S. typhimurium* is infected with phage P22 in the presence of spermine; resuspension of the infected *S. typhimurium* in spermine-free medium allows the burst to occur normally (10); this result indicates that the inhibition is reversible.

We confirmed the findings of Kay on the inhibition of the burst of bacteriophage 3 by spermine, but found that this can be done only if highly purified preparations of spermine are used. Some commercial preparations contain an unidentified contaminant which can counteract the inhibition of the bacteriophage 3 burst by spermine. This material is not separated by recrystallization; its removal may be accomplished by chromatography on Dowex 50.

Polyglucose sulfate treatment causes the release of over 50% of the T2 phage polyamines into the suspending medium and a parallel inactivation of the phage (297, 471). A causal relationship, however, has not been established; indeed it seems more likely that both effects result from damage to the phage coat by polyglucose sulfate. If the phage coat were not damaged, polyamines would not be released from the phage, since, as we have already discussed, T2 bacteriophages are impermeable to labeled polyamines. Furthermore, addition of polyamines does not reverse the inactivation caused by polyglucose sulfate (334).

Antagonism of spermine to anti-phage sera. Either polyamines (spermine, 1,4-diaminobutane, or 1,5-diaminopentane) or polyanions (polyglucose sulfate) decrease the inactivation of T2-bacteriophage by anti-T2 serum (296), presumably by interfering with the ionic interaction of the antigen and the antibody (138). This system can also be used to demonstrate the interaction of polyamines and polyglucose sulfate with each other. Certain concentrations of spermine, for example, decrease the inactivation by polyglucose sulfate of anti-T2 serum, and, contrariwise, certain concentrations of polyglucose sulfate can decrease the spermine inactivation of the anti-T2 serum. There is no evidence that these effects are specific for the polyamines; they presumably could be shown with other polybasic compounds.

Stimulation of phage ϕR development. Tucker (433) has reported a low yield of phage ϕR from *E. coli* C unless a divalent cation or a polyamine is present during the latter part of the latent period. The effect is on phage maturation rather than lysis. 1,3-Diaminopropane and agmatine are the most effective amines, followed by spermidine, 1,2-diaminopropane, and spermine; 1,4-diaminobutane is only slightly active.

VI. EFFECT OF POLYAMINES ON MISCELLANEOUS ENZYME ACTIVITIES

Spermine affects the activity of several enzymes. In general, however, the mechanism of the spermine effect is obscure. In some cases the action seems to be

on the substrate, *i.e.*, by binding an acidic substrate, or by competing with a basic enzyme (such as lysozyme or ribonuclease) for acidic sites on a substrate. In other cases, one may postulate an effect of the polybasic spermine on the configuration of enzymes or the aggregation of subunits. In addition, with crude enzyme preparations the polyamines may have an indirect effect by influencing the release of enzymes from particles or cells.

Lysozyme. In section II we discussed the stabilization of spheroplasts by the addition of spermine *after* the spheroplasts had been prepared by the action of lysozyme. In addition, there is some evidence that spermine can inhibit the action of the lysozyme directly. Thus, Wright (462) described a strain of *Bacillus anthracis* whose growth is inhibited by lysozyme. Spermine counteracts this effect and allows growth in the presence of the enzyme. Brown (62, 63) has demonstrated that spermine inhibits the autolytic action of a lysozyme-like enzyme which occurs in cell wall preparations of a marine bacterium. With *E. coli* the addition of spermine simultaneously with or before the addition of lysozyme appears to inhibit partially the action of lysozyme. Thus, in the presence of spermine, lysozyme causes little change in the morphology of the bacteria (*i.e.*, few spheroplast forms are observed), and there is a decrease in the bactericidal action (402). With *M. lysodeikticus*, on the other hand, spermine does not affect the ability of lysozyme to form spheroplasts, as assayed either with the electron microscope or by the release of hexosamine-containing fragments (146). Similarly spermine does not inhibit the degradation by lysozyme of the cell walls of a species of *Micrococcus* (62).

Dihydrofolate reductase. Spermine (0.06 M) activates dihydrofolate reductase 3-fold (288). Similar stimulation occurs with higher concentrations of spermidine, 1,4-diaminobutane, 1,5-diaminopentane and other basic compounds. At even higher concentrations (1 M) these substances inhibit the dihydrofolate reductase activity. The mechanism of these effects is not known.

β-Glucuronidase, highly purified from calf spleen or liver, is inactivated by dilution. This inactivation is reversed by spermine, spermidine, and various diamines (37).

Tetrahydrofolic acid formylase. Spermine stimulates 2- to 4-fold the rate of formyltetrahydrofolate formation catalyzed by purified tetrahydrofolate formylase from *Lactobacillus arabinosus* and *L. casei* (251, 251a). This effect of spermine is due to an increase in the affinity of the enzyme for the substrate. This finding may account for the observations of this same group that spermine (a) stimulates the utilization of formate by *L. arabinosus*, (b) decreases the growth requirement of *Pediococcus cerevisiae* for 5-formyltetrahydrofolate, (c) decreases the growth requirement of *Lactobacillus leichmannii* for 5-formyltetrahydrofolate, thymidine, or thymine, (d) decreases the amount of thymidine required for reversal of the growth inhibition of *L. arabinosus* caused by an inhibitory concentration of 2,4-diamino-6,7-diphenylpteridine, aminopterin, or sulfanilamide, and (e) reverses the toxicity of the thiobenzylester of *p*-aminosalicylic acid for *Streptococcus lactis* (385, 435).

Uridinediphosphogalactose-4-epimerase. With yeast uridinediphosphogalactose-

4-epimerase, spermine and spermidine increase the reaction velocity at low substrate concentrations. Experiments with spermine indicate that this amine both decreases the K_m and increases the maximal velocity (V_{max}) (86).

Prostatic acid phosphatase is inactivated by dilution in saline. Polyamines in low concentrations protect against this inactivation, the protective activity increasing with the chain length of the compound (194). Acetylation of the amino groups of spermine or treatment with nitrous acid markedly reduces the protective action. The nature of the protective groups is unclear, however, since modification of the amino groups by treatment with benzylchloride, 1-chloro-2,4-dinitrofluorobenzene, or formaldehyde has little or no effect on the protective activity.

Cytidine monophosphate → deoxycytidine monophosphate. This enzymatic activity is increased 2-fold when 5×10^{-3} M spermidine, spermine, or streptomycin is added to the medium used to extract the activity from T_6r^+-infected *E. coli* (77).

Miscellaneous. Purified hexokinase and pyruvic carboxylase are inhibited by 0.05 M spermine. These effects may account for the inhibition by spermine of CO_2 production from glucose in whole yeast or yeast extracts (351). Spermine activates muscle phosphorylase (351) and bovine testicular hyaluronidase (292). Spermine or several synthetic polyamines have been reported to decrease the time required for the reduction of methylene blue by either aged *E. coli* cells or washed *S. aureus* suspensions in the presence of glucose, pyruvate, or succinate (40, 326); several synthetic polyamines (but not spermine) also increased the oxidation of pyruvate by aged *E. coli* (40). Evans *et al.* (113) showed that spermine causes an inhibition of oxygen uptake when guinea pig brain brei is incubated with glucose, lactate, or pyruvate, but not with glutamate or succinate. A small inhibition of the activity of chymotrypsin (378) has also been reported. Slight activating and inhibitory effects of spermine on trypsin (63, 204) have been described.

Spermine stimulates the accumulation of the exoenzyme, amylase, by resting cells of *B. subtilis* (470). A report (326) that enzyme induction is inhibited by spermine cannot be considered conclusive since the effects may be due to (a) a direct action of the polyamine on the enzyme assay or (b) toxicity of the polyamine for the bacteria that are being induced.

VII. OTHER PHARMACOLOGIC EFFECTS OF POLYAMINES

A. Antimicrobial effects

Growth of several bacteria and yeast is inhibited by the polyamines, but the sensitivities of different species vary markedly (39, 139, 147, 149, 323, 326, 345, 347, 383, 407). At pH 7 *S. aureus* is killed by spermine at 5×10^{-4} M, while 0.01 M is necessary to kill *E. coli*. The bactericidal potency increases about 10-fold when the pH of the medium is raised from 7 to 8. There is some evidence that an actively metabolizing cell is necessary for this effect, since Razin and Rozansky (326) have shown that glucose and a temperature of 37° are required for the

bactericidal action against *S. aureus*. This action of spermine accounts for the earlier observations on the bactericidal action of human semen (149, 325, 346).

The antibacterial action of spermine can be antagonized by high concentrations of 1,4-diaminobutane, inorganic cations, nucleic acid, mononucleotides, lecithin, and basic amino acids (39, 147, 323, 326). Some of these materials may account for the antagonistic effect of yeast extract on the antibacterial action of spermine (407).

An interesting example of an antibacterial effect due to interference with permeability mechanisms has been presented by Mandelstam (276, 277). 1,5-Diaminopentane does not inhibit growth of wild-type *E. coli* but is bacteriostatic in a lysine-requiring mutant of *E. coli*. He showed that the diamine prevents the uptake of the essential basic amino acid and thereby prevents growth.

B. Toxic effects of enzymatic oxidation products of spermine and spermidine

Concentrations of spermine that do not damage the cells or organisms may become very toxic when mixed with beef plasma amine oxidase (see section X); presumably the products of the enzymatic oxidation of spermine are the toxic factors. This observation was first made by Hirsch and Dubos in their studies on *Mycobacterium tuberculosis* (128, 175–180). Subsequently, similar effects were observed with *E. coli* (407), *S. aureus* (407), *Trypanosoma equiperdum* (407), the T-odd series of *E. coli* bacteriophages (31), mammalian spermatozoa (407), and a variety of mammalian and chick cell lines in cell culture (6, 150, 258). Similarly oxidized spermidine is much more lethal than spermidine for spermatozoa, bacteria, and bacteriophages.

C. Effects of polyamines in animals

Kidney toxicity. In the first work on polyamines in our laboratory, Rosenthal *et al.* (342) showed that spermine has a marked renal toxicity. Parenteral administration of 0.075 to 0.15 mmol of spermine per kg to a variety of animals (mouse, rat, guinea pig, rabbit, dog) produces only relatively mild acute effects, but proteinuria and serum nonprotein nitrogen retention gradually develop, followed by death from renal failure within a week. Animals receiving considerably less spermine have an early diuresis and a moderate proteinuria, but no permanent renal damage. Spermidine is much less toxic than spermine and 1,4-diaminobutane is not toxic at comparable doses. In contrast to the effect of parenteral spermine, only a slight transient proteinuria results from the administration of large amounts of spermine in the diet (407). In man, vomiting, albuminuria, hematuria, acetonuria, azotemia, and hyperglycemia are observed after intramuscular injection of spermine [0.033 mmol per kg (333)].

The kidneys of animals that die after a toxic dose of spermine show marked necrosis of the epithelial lining of the proximal convoluted tubules without any inflammatory changes (125, 342). The glomeruli, distal convoluted tubules, collecting tubules, and blood vessels appear normal.

Administration of spermine directly into the renal artery of rabbits produces extensive glomerular and tubular necrosis of the corresponding kidney, and

eventually extensive scarring. Numerous juxtaglomerular cells appear in the injected kidney, and hypertension develops. The uninjected kidney escapes damage and undergoes compensatory hypertrophy (405, 406). Although spermidine does not produce as much renal damage as spermine when injected subcutaneously or intraperitoneally, both polyamines are equally toxic when injected into the renal artery.

The mechanism of the renal toxicity is not known. It is possible that spermine is concentrated within the renal cells and binds nucleic acids, phospholipids, or other acidic constituents. Alternatively, the spermine may be converted within the kidney to a toxic product. Whether spermine is degraded by the kidney is not known, but by analogy with other enzymatic systems for spermine oxidation one may postulate the formation of an aminoaldehyde that either is toxic itself or is converted to a toxic product, such as acrolein (see section X).

Miscellaneous effects. Several other effects of spermine have been described. These include a transitory fall in blood pressure (407, 458), slowing of the pulse and respiration (458), labored respiration (407), a prolongation of the hypoglycemic effect caused by zinc insulin (124), and an acute diuresis (407). Spermine has been reported, on the one hand, to have antihistamine properties (4, 191, 242) and, on the other hand, to be a histamine releaser (8); spermine also prolongs and increases the effects of administered histamine (311). The diamines are relatively nontoxic, but at very high doses a variety of toxic effects has been described (130, 148).

Boyland (53) demonstrated that spermine, 1,4-diaminobutane, 1,5-diaminopentane, and several other bases can inhibit the growth of several tumors in mice. A slight inhibition of the growth of a Yoshida sarcoma *in vitro*, but not of an Ehrlich solid tumor *in vivo*, has been observed with spermine and spermidine (291). Spermine in the absence of beef serum amine oxidase is not toxic to several mammalian tumor cells in cell culture (6, 150).

Spermine has two different effects on blood coagulation: (a) like protamine, spermine (but not spermidine or 1,4-diaminobutane) decreases the clotting time of heparinized blood (407); (b) spermine in high concentrations prolongs the coagulation time of unheparinized blood (407). Spermine also affects the coagulation times of fibrinogen-thrombin mixtures; low concentrations cause a small decrease in the coagulation time, while higher concentrations result in a marked increase (9). The mechanisms of these effects are not known. There is some evidence that the polyamines can form complexes with the acidic heparin and thrombin molecules. Complex formation between heparin and the polyamines is indicated by the precipitation that occurs when spermine, spermidine, 1,4-diaminobutane, or 1,5-diaminopentane is added to a solution of heparin in aqueous alcohol (8, 445). The observations that polyamines can dissociate a heparin-histamine complex (8, 211), that heparin prevents the stabilizing action of spermine on *P. tularensis* (268), and that spermine decreases the staining of mast cell granules with toluidine blue (19) afford further evidence for the affinity of polyamines for heparin. Complex formation between spermine and thrombin has also been postulated on the basis of the decreased solubility of purified thrombin in the presence of the amines (9).

D. Antagonism of polyamines to various basic antimicrobial compounds (quinacrine, proflavine, diamidines, and streptomycin)

The polyamines antagonize the action of a variety of antimicrobial agents. Since these are all basic compounds, the polyamines may act by competing with the agents either for entry into the cell, or for some essential acidic compounds within the cell, such as nucleic acids.

Quinacrine. One of the first indications of a possible biological action of the polyamines was the work of Silverman and Evans (382, 383) and of Miller and Peters (286) on the ability of spermine, spermidine, or several related synthetic polyamines to antagonize the antibacterial action of quinacrine and quinine. As in many other instances in which polyamines are active, divalent cations (Ca^{++}, Mg^{++}, Mn^{++}, or Ba^{++}) can also overcome the bacteriostatic effect of quinacrine (380, 381); certain monoamines and diamines are inactive. Spermine also prevents the inhibition of respiration of yeast cells that is caused by quinine, as well as by protamine, methylene blue, crystal violet, or trypaflavine (285).

Proflavine. The effect of spermine on the inhibition of bacteriophage by proflavine has been studied by two groups with somewhat different results. Fraser and Mahler (132) studied the inactivating effect of proflavine on isolated bacteriophages (T2, π, and ϕX-174); some inactivation occurs in the dark, but considerably more in the light ("photosensitization"). The diamines antagonize both the light and dark inhibition. Kay (201) studied burst size in *E. coli* infected with phage 3, and reported that proflavine inhibits phage multiplication. Spermidine, agmatine, or 1,4-diaminobutane prevents this inhibition.

Diamidines. Spermidine and spermine antagonize the inhibition of *E. coli* growth caused by propamidine (286, 391). Inhibition of the growth of *E. coli* and of *S. aureus* by stilbamidine or pentamidine is overcome by high concentrations of diethylenetriamine, triethylenetetramine, and tetraethylenepentamine, although spermine is not effective (39). Bichowsky-Slomnitzki (40) found that several synthetic polyamines prevent the inhibition by diamidines of respiration, glycolysis, and several dehydrogenase activities in intact *E. coli* and *S. aureus* cells. Spermine also reduces the inhibitory effect of pentamidine on the incorporation of C^{14}-amino acids into thymus nuclei *in vitro* (13).

Relationship of polyamines to streptomycin effects. Several reports have been published on the ability of polyamines to antagonize certain effects of streptomycin. The polyamines have been shown to prevent (a) the inhibitory effect of dihydrostreptomycin on the induction of β-galactosidase in *E. coli* suspensions (54); (b) the increase in the accumulation of pyruvate that occurs when dihydrostreptomycin is added to exponentially growing *E. coli* (54); (c) the inhibitory effect of dihydrostreptomycin on a soluble succinate-triphenyltetrazolium dehydrogenase from *E. coli* (55); and (d) the effect of streptomycin in inhibiting DNA injection by streptomycin-sensitive bacteriophages of *Streptococcus faecium* (61). In several of the systems the action of the streptomycin also could be antagonized by Mg^{++}. In addition, in one laboratory (269) [but not in another (126)] spermidine or spermine partially prevented the inhibitory effect of streptomycin on

the incorporation of amino acids into polypeptide or protein in a cell-free system from *E. coli*. Mager *et al.* (269) suggested that in these experiments streptomycin may be acting in part, by interfering with the postulated function of Mg^{++} and polyamines in preserving the integrity of the ribosomes (section III).

Sevag and Drabble (372) have reported that the addition of spermine or spermidine to the growth medium prevents the emergence of resistant cells of *S. aureus* and of *Aerobacter aerogenes*, when sensitive strains of these organisms are treated with streptomycin, penicillin, or several other antibiotics. These results are surprising since they are not consistent with our present knowledge of the action of various antibiotics. Although these authors suggest that the polyamines may be behaving as antimutagenic agents, various other more trivial explanations, such as potentiation of the inhibitory action of the antibiotic by spermine, should be excluded. More detailed data must be obtained with this and other systems before such an important conclusion is accepted.

VIII. BIOSYNTHESIS

Biosynthesis of spermidine and spermine in microorganisms. A summary of the various steps involved in the biosynthesis of spermidine is presented in figure 1.

The first experiments on the biosynthesis of spermidine and spermine were carried out with growing cultures of *E. coli* and *A. nidulans*, using either C^{14}-labeled ornithine or C^{14}-N^{15}-labeled 1,4-diaminobutane (417, 419). These experiments confirmed the expected conversion of ornithine to 1,4-diaminobutane, and showed that the diaminobutane component of spermidine and of spermine is derived directly from 1,4-diaminobutane. Greene (144), in our laboratory, then demonstrated that the aminopropyl unit is derived from methionine. When *N. crassa* is grown on C^{14}-methionine (labeled in the α-carbon atom), this label is incorporated into spermidine (28, 144).

The biosynthesis of spermidine was then demonstrated in cell-free extracts of *E. coli* (418, 419). From this source three enzymes have been partially purified that carry out reactions 1, 2, and 3 of figure 1. Reaction 1 represents the formation of adenosylmethionine from ATP and methionine. This reaction had been described previously (68) in yeast and liver as the first step in methylation reactions; the methyl group of adenosylmethionine is transferred to a variety of substrates, such as guanidoacetic acid, nicotinamide, histamine, and noradrenaline. For the biosynthesis of spermidine, however, S-adenosylmethionine is first decarboxylated (reaction 2), and then the aminopropyl moiety is transferred to 1,4-diaminobutane (reaction 3). This reaction is analogous to transmethylation, except that the aminopropyl chain, rather than the methyl group is transferred from the sulfonium group.

Reaction 1. The *E. coli* preparation catalyzing this reaction has been purified 1500-fold. Experiments with ATP^{32} showed that the orthophosphate arises from the terminal phosphate of ATP and the pyrophosphate from the proximal two phosphate groups (420, 423). Although these isotope data exclude an intermediary role of free tripolyphosphate, unpublished work in our laboratory has shown that the purified enzyme still has a significant tripolyphosphatase activity, which is

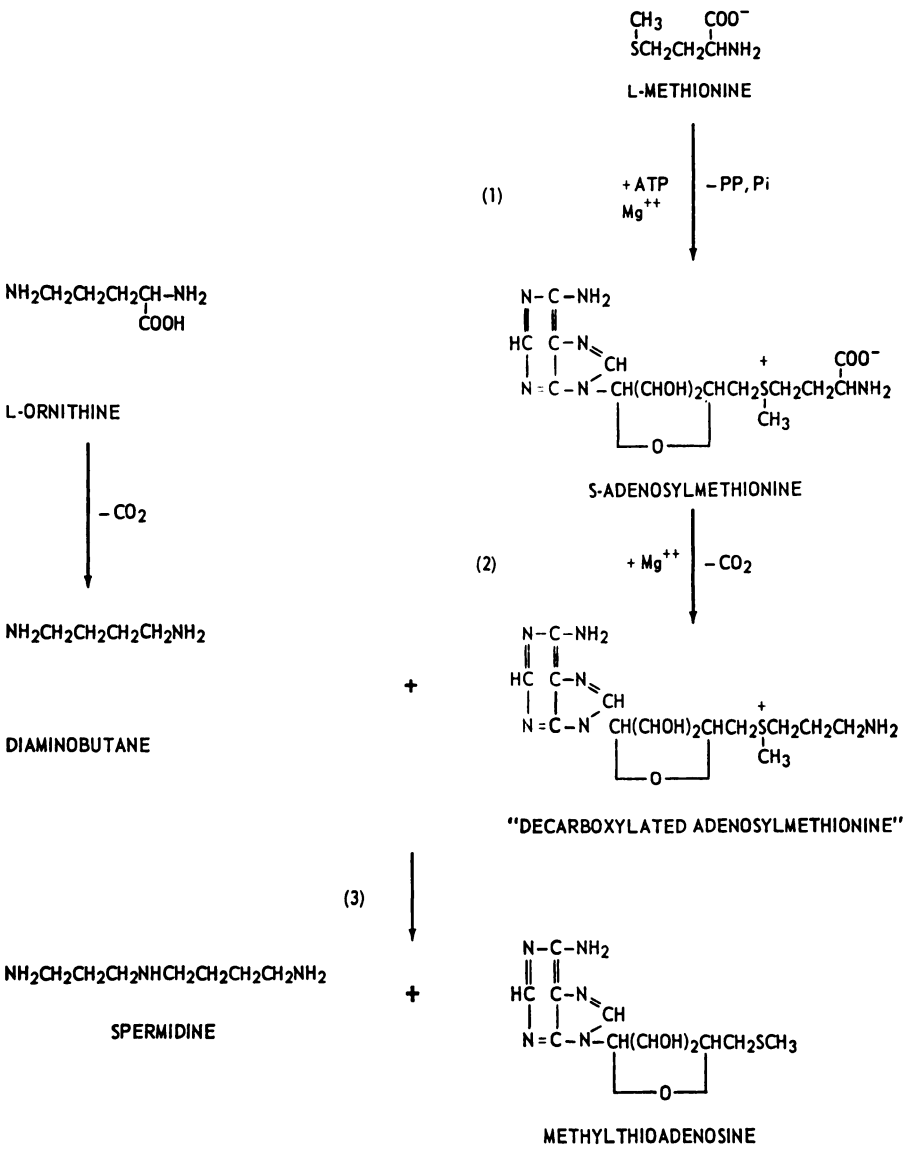


FIG. 1. Biosynthesis of spermidine in *Escherichia coli*.

increased by S-adenosylmethionine; it is possible, therefore, as indicated by Mudd (303), that an enzyme-bound tripolyphosphate is an intermediate. Thus, this enzyme is similar to those previously described in purified liver and yeast preparations by Cantoni, Durell, and Mudd (68, 303).

Reaction 2. The enzyme from *E. coli* extracts that catalyzes the decarboxylation reaction has been purified 20- to 50-fold (403). It requires Mg^{++}, and is inhibited by cyanide. The product of the reaction, "decarboxylated adenosyl-

methionine," can be purified by chromatography on Dowex 50. "Decarboxylated adenosylmethionine" has also been synthesized chemically by Jamieson (193).

Reaction 3. The enzyme from *E. coli* extracts that catalyzes reaction 3 has been purified about 1000-fold (404). Either the product of reaction 2 or a chemically synthesized "decarboxylated adenosylmethionine" can be used as a substrate for reaction 3. This enzyme preparation catalyzes the synthesis only of spermidine; no synthesis of spermine can be found under the conditions tested. The other product of reaction 3 is methylthioadenosine, which can be isolated if a highly purified enzyme is used; with crude bacterial extracts methylthioadenosine is rapidly metabolized (373, 420, 422). Although our main interest in reaction 3 has been the enzymatic biosynthesis of spermidine, it is noteworthy that this reaction affords a mechanism for the biosynthesis of methylthioadenosine. Schlenk and de Palma (357) showed that most (but not all) of the methylthioadenosine isolated by the earlier methods had been formed by the decomposition of S-adenosylmethionine during the isolation procedure. The results reported above, as well as the studies of Mudd (302) and of Shapiro and Mather (373) on the conversion of S-adenosylmethionine to methylthioadenosine by yeast and bacteria enzymes, demonstrate that thiomethyladenosine can be formed enzymatically and is not solely a degradation product of adenosylmethionine.

Biosynthesis of spermine and spermidine in animals. No evidence for the biosynthesis of polyamines in mammals has been reported, except for a preliminary report of a small incorporation of C^{14}-N^{15}-1,4-diaminobutane into the polyamine fraction of minced rat prostate (417). The presence of polyamines in animal tissues does not indicate biosynthesis, since it is possible that animals derive their polyamines either from the diet or from the intestinal flora. Analyses showing the presence of polyamines have been carried out on germ-free animals (313, 343), but these animals had been on a regular diet. Assays have also been carried out with conventional animals on a purified diet (343), but no polyamine analyses have been carried out on germ-free animals that had been on a purified diet.

The formation of spermine and spermidine from C^{14}-1,4-diaminobutane and from C^{14}-methionine has been demonstrated in developing chick embryos by Raina (319, 320).

Biosynthesis of diamines. Two pathways have been described for the biosynthesis of *1,4-diaminobutane*: (1) L-Ornithine is decarboxylated by ornithine decarboxylase (22, 81, 137, 295, 320, 337). (2) L-Arginine is decarboxylated by arginine decarboxylase to form agmatine. Agmatine can be hydrolyzed directly to 1,4-diaminobutane and urea, or it may first form an intermediate, N-carbamyl-1,4-diaminobutane (137, 260, 295, 328, 386, 387, 388, 476).

Most of the studies on ornithine and arginine decarboxylases have been in bacteria (137). These enzymes have also been described in plants, and Smith has reported a large increase in the level of arginine decarboxylase in potassium-deficient plants (386). This increase in arginine decarboxylase may account for the high concentrations of agmatine and 1,4-diaminobutane in a variety of potassium-deficient plants (81, 82, 330, 386, 388).

1,5-Diaminopentane is formed by the action of lysine decarboxylase on

L-lysine (137). This enzyme is present in bacteria, but has not been described in plant or animal tissues. No information is available on the biosynthesis of *1,3-diaminopropane* even though this amine is present in some bacteria (section I-B). The most likely pathway is decarboxylation of α,γ-diaminobutyric acid. However, it is possible that 1,3-diaminopropane is formed by the bacterial degradation of spermidine (to be discussed in section X).

IX. UPTAKE OF POLYAMINES BY VARIOUS CELLS

Bacteria and yeast. The polyamine content of bacteria and yeast cells can be affected by the concentration of polyamines in the external medium as well as by biosynthesis. The uptake of polyamines from the medium has been shown in both growing cultures and nongrowing suspensions of a variety of organisms (28, 103, 323, 326, 341, 419, 443).

Cells can take up amines whether or not they are normal constituents of the cell. *E. coli*, for example, grown in media containing spermine, contains large amounts of spermine, diacetylspermine, and monoacetylspermine but little spermidine or 1,4-diaminobutane; without the addition of spermine to the culture medium, only spermidine, 1,4-diaminobutane and traces of acetylspermidine and acetyl-1,4-diaminobutane are present in the cells. The uptake of spermine by *E. coli* thus not only increases the quantity of spermine and its derivatives in the cell, but also decreases the concentration of the other amines in the cell. There are two possible explanations for this effect: (a) added spermine represses or inhibits the enzymes that normally synthesize 1,4-diaminobutane and spermidine, and (b) these amines are synthesized normally, but are displaced from their normal binding sites by spermine. In support of the latter possibility is the observation that, when *E. coli* containing C^{14}-spermidine is suspended in a medium containing spermine, the spermine enters the cells and most of the C^{14}-spermidine is displaced into the medium (103).

"Active uptake" systems have recently been found in *E. coli* for the accumulation of C^{14}-1,4-diaminobutane and C^{14}-spermidine (421). "Active uptake" only occurs at 37°, requires glucose, is inhibited by 0.005 M dinitrophenol, and does not occur in heat-killed or toluenized cells. With C^{14}-spermine two types of uptake occur: (1) An "active uptake" system that requires active bacterial metabolism; the accumulated C^{14}-spermine is not washed out when the bacteria are washed with medium containing C^{12}-spermine. (2) A large "nonspecific" adsorption that does not require active metabolism; the adsorbed C^{14}-spermine is easily removed by C^{12}-spermine washes. With *S. aureus* Razin and Rozansky (326) found only the latter type of adsorption, since the same amount of spermine was taken up by bacteria at 37° or at 4° or by heat-killed bacteria.

Blood cells. After the intravenous administration of spermine to rabbits, approximately one-fourth of the injected dose is present in the blood after 1 hour; two-thirds of this is in the formed elements while one-third is in the plasma. Five hours after the injection, the plasma contains essentially no spermine, while the amount of spermine in the cells is still twice the normal value (343). No studies of the uptake of spermine by blood cells *in vitro* have been published.

X. METABOLISM OF POLYAMINES

A. Oxidation of spermidine and spermine

1. *Animal preparations.* The only known enzyme in animal tissues that actively carries out the oxidative deamination of spermine or spermidine is a soluble plasma amine oxidase (177, 178). This enzyme has been purified (141, 350, 411, 437) and crystallized (464) from beef plasma, and carries out the following reactions (178, 409, 411):

$$NH_2CH_2CH_2CH_2NH(CH_2)_4NHCH_2CH_2CH_2NH_2 + 2\ H_2O + 2\ O_2 \rightarrow$$
$$OHCCH_2CH_2NH(CH_2)_4NHCH_2CH_2CHO + 2\ NH_3 + 2\ H_2O_2.$$

$$NH_2CH_2CH_2CH_2NH(CH_2)_4NH_2 + H_2O + O_2 \rightarrow$$
$$OHCCH_2CH_2NH(CH_2)_4NH_2 + NH_3 + H_2O_2$$

Although spermidine and spermine are oxidized most rapidly, a number of other amines are also oxidized, including benzylamine, homosulfanilamide, phenethylamine, and kynuramine, as well as several aliphatic monoamines and decamethylenediamine (411, 447, 464). Benzylamine is a particularly useful substrate, since it is oxidized to benzaldehyde, the formation of which can be followed spectrophotometrically at 250 mμ (411). The relative rates of reaction with various amines are essentially the same for both crude and purified preparations. No significant oxygen uptake is obtained with tryptamine, 5-hydroxytryptamine, norepinephrine, epinephrine, histamine, 1,4-diaminobutane, or 1,5-diaminopentane (411, 464). Using the crystalline preparation, no oxygen uptake is obtained with tyramine or mescaline (464). The substrate specificity of this enzyme is thus different from that of tissue monoamine oxidase[6] or diamine oxidase.

The species distribution of this amine oxidase is surprising. The oxidation of spermidine and spermine was first described in the plasma of sheep and cattle by Hirsch (177, 178); subsequently Blaschko and co-workers (45–48) reported that this activity is also present in the plasma of a number of other ruminants and in some nonruminants. The amount of spermine oxidase in plasma varies with age. Blaschko found that little, if any, spermine oxidase is present in the sera of newborn goats, but that the levels increase gradually during the neonatal months (46). In all of these animals the plasma also contains benzylamine oxidase activity. On the other hand, the plasmas of many other species contain only benzylamine oxidase activity, and have no activity against spermidine or spermine (36, 45–47, 64).

Yamada *et al.* (463) have reported that the molecular weight of the crystalline plasma amine oxidase is about 250,000, and have studied several other physicochemical properties of this preparation. Copper is a constituent of the crystalline enzyme (465, 466, 468), although zinc has been reported for a less purified preparation (140). Evidence for the presence of pyridoxal has been presented, but this

[6] Tissue monoamine oxidases have recently been reviewed by H. Blaschko, in The Enzymes, ed. by P. Boyer, H. Lardy, and K. Myrbäck, 2nd ed., vol. 8, pp. 337–351. Academic Press, Inc., New York, 1963.

is not conclusive (465, 467). A pyridoxal phosphate cofactor would be consistent with earlier observations on the inhibition of the enzyme by isonicotinic acid hydrazide and isopropylisonicotinic acid hydrazide, and such carbonyl binding agents as cyanide, semicarbazide, and hydroxylamine (411). Other inhibitors have also been described, including Dibenamine, Pyribenzamine, Benadryl, and several amidines (49, 411).

The aldehyde products of the plasma amine oxidase reaction are of particular interest because of their toxicity for various bacteria, bacteriophages, and tissue culture cells (see section VII-B). The aldehyde products themselves are rather unstable and attempts to isolate them directly have been unsuccessful (411, 437). We have recently characterized them by reduction with sodium borohydride and comparison with the corresponding synthetic alcohols (409). Some attempts at chemical synthesis of the aldehydes have been made by Carvajal (69, 70), but these products have not yet been characterized.

We have recently found that the dialdehyde formed by the oxidation of spermine decomposes upon heating to 1,4-diaminobutane, presumably by a nonenzymatic β-elimination reaction (410); spermidine is formed by a comparable decomposition of the intermediate monoaldehyde. These findings explain earlier observations indicating that spermine is oxidized to 1,4-diaminobutane by plasma amine oxidase, and that spermidine is an intermediate in this reaction (27, 407, 437). The other product of the decomposition would be acrolein, and this would explain the recent findings of Alarcon (7) that acrolein can be detected after heating and distillation of the oxidation products of spermine. There is no evidence (409) that any spermidine occurs as an intermediate in the enzymatic oxidation of spermine by purified plasma amine oxidase, or that any significant amounts of 1,4-diaminobutane, or 1,3-diaminopropane are formed as primary products of spermine or spermidine oxidation.

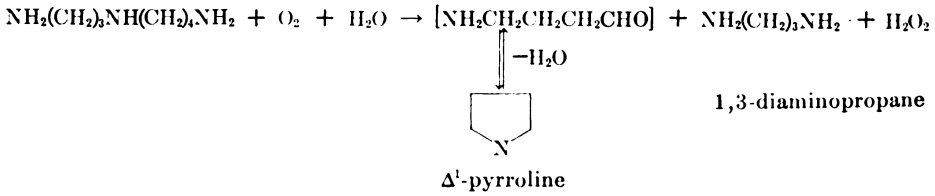
The oxidation of spermine by other animal tissues has been shown indirectly by the formation of a tuberculostatic factor upon incubation of spermine with extracts of guinea pig kidney (177). Some oxidation of these polyamines by a relatively crude preparation of diamine oxidase has been reported (161, 473) with the formation of N-(3-aminopropyl)-pyrroline (161), but no significant oxidation was found with a purified preparation (199). There is no evidence at present for the oxidation of spermidine or spermine by the particulate monoamine oxidase of tissues (411).

Some evidence for the oxidation of spermine to spermidine *in vivo* has been found. When spermine is administered parenterally to rats, mice, or rabbits, up to 20% of the dose administered appears in the urine unchanged, while 4 to 8% is excreted as spermidine. When spermidine is injected into mice, 18% of the administered dose is excreted unchanged, and no spermine appears in the urine (343). Experiments with labeled amines will be necessary to show definitely whether the spermidine which is excreted after spermine administration is derived from the injected amine or whether it is released from tissue stores.

2. *Bacterial preparations.* Oxidative degradation of spermidine and spermine by bacterial preparations was first observed by Silverman and Evans (383),

using whole cells or lyophilized preparations of *Pseudomonas pyocyaneae*. Subsequently, oxidation of the polyamines has been observed with intact cells or crude extracts of *Mycobacterium smegmatis*, *N. perflava*, *Pseudomonas aeruginosa*, and *S. marcescens* (30, 322, 324, 344, 443, 444). The oxidative activities for these amines usually increase when the organisms are grown with the amines in the growth media, indicating that the relevant enzymes are "inducible."

Since these studies were carried out with intact cells or crude extracts, few conclusions could be drawn on the substrate specificity of the enzymes or on the nature of the products. Some data were available, however, in the studies with *N. perflava* and *S. marcescens*, showing that one of the products of spermidine oxidation is 1,3-diaminopropane (324, 444). The latter finding has been defined further for *Serratia marcescens* by recent studies in our laboratory by Bachrach (25, 26), in which he purified an enzyme that carries out the following reaction:



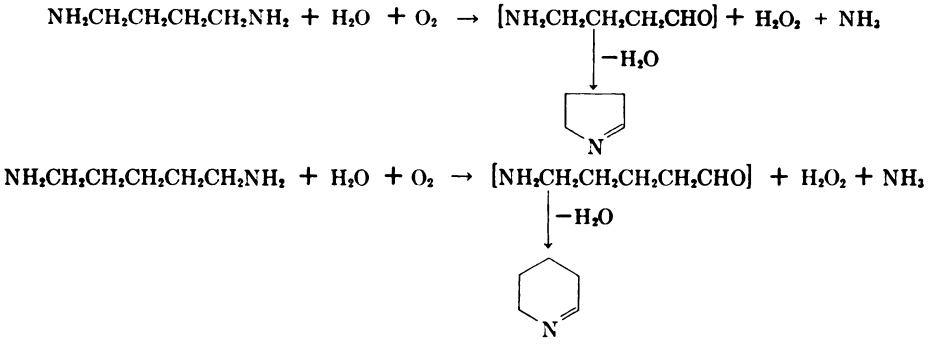
This enzyme oxidizes spermidine and its homologue bis-(3-aminopropyl)-amine, but not spermine or any of the diamines or monoamines tested. With more purified preparations, oxidation of spermidine is markedly stimulated by an electron carrier, such as phenazinemethosulfate (66). Δ^1-Pyrroline, which is formed by the oxidation of spermidine (but not by the oxidation of bis-(3-aminopropyl)-amine), reacts with *o*-aminobenzaldehyde to give a yellow color (182, 361). These reactions are the basis of a specific enzymatic assay for spermidine (29).

3. *Plant preparations.* Spermidine and spermine, as well as the short-chain diamines, are oxidized by crude or purified plant diamine oxidase preparations (174a, 278, 448). Most of the studies on this enzyme, however, have been carried out with various diamines as substrates (see below), without any indication of whether spermidine or spermine is also oxidized. The oxidation products of spermine and spermidine have not been identified with certainty. Hasse and Schührer (161) have presented evidence that oxidation of either spermine or spermidine yields N-(3-aminopropyl)-pyrroline, presumably from the cyclization of the postulated primary product of spermidine oxidation, $NH_2CH_2CH_2CH_2 \cdot NHCH_2CH_2CH_2CHO$. Since these results were obtained with crude enzyme preparations, requiring prolonged incubations, further work is needed to establish the nature of the products.

B. Oxidation of diamines

1. *Animals.* The oxidation of 1,4-diaminobutane, 1,5-diaminopentane, and various other diamines by animal diamine oxidase has been extensively investigated by Zeller and others. This area has been reviewed previously (413, 474,

475), and therefore will be discussed only briefly here. Diamine oxidase oxidizes 1,4-diaminobutane and 1,5-diaminopentane to the corresponding aminoaldehydes, which then cyclize to Δ^1-pyrroline and Δ^1-piperideine, respectively (161, 182, 396, 412):



Diamine oxidase activity is present in a variety of animal tissues, and has been purified from hog kidney (20, 200, 397, 412, 475). The purified preparations, however, have considerably lower specific activities than the purified plant amine oxidase discussed below. The cofactors of animal diamine oxidase have not been identified, although some evidence has been presented for both flavin adenine dinucleotide and pyridoxal phosphate (87, 142, 199, 200, 475).

There has been disagreement on whether "diamine oxidase" is the same enzyme as "histaminase" (*i.e.*, whether the same enzyme oxidizes both diamines and histamine) (200). Zeller (475) has recently reviewed this problem, and has concluded that only one enzyme is involved.

Studies on the oxidation of C^{14}-1,5-diaminopentane *in vivo* have been carried out by Schayer *et al.* (356). In 4 hours 36% of an injected dose is excreted as $C^{14}O_2$; this is 95% inhibited by the administration of aminoguanidine, a diamine oxidase inhibitor. Evidence for the formation of cyclic products *in vivo* during 1,5-diaminopentane oxidation is afforded by the experiments of Nordenström (307) who showed the excretion of piperidine by rabbits after the administration of 1,5-diaminopentane. In rats, δ-aminovaleric acid is found in the urine after the subcutaneous administration of 1,5-diaminopentane (90).

2. *Bacteria. Diamine oxidation* has been described in *M. smegmatis*, *P. aeruginosa*, *Pseudomonas fluorescens*, *S. marcescens*, *Corynebacterium pseudodiphtheriticum*, *Mycobacterium avium*, *M. tuberculosis*, *P. pyocyaneae*, *Micrococcus pyogenes* var. *aureus*, *Achromobacter* sp., *A. aerogenes*, and *E. coli* (136, 192, 293, 310a, 324, 344, 354, 446). The metabolism of diamines seems to be "inducible" since it is increased by the addition of the substrate to the culture medium. The first step in the metabolism of the diamines by these cells has usually been assumed to be an oxidative deamination of the diamine oxidase type. Recently, however, Kim and Tchen (*cf.* section X-C) have shown that in extracts from 1,4-diaminobutane-adapted *E. coli* the first step is a transamination to a keto-acid catalyzed by pyridoxal phosphate (208, 208a, 209). Further work is necessary

to evaluate which of these two reactions (*i.e.*, transamination or diamine oxidase action) is responsible for the metabolism of diamines observed with other bacteria. The γ-aminobutyraldehyde formed by either transamination or oxidation can undergo either reversible cyclization to Δ^1-pyrroline or further oxidation to γ-aminobutyric acid followed by transamination to succinic semialdehyde and oxidation to succinic acid (24, 30, 192, 208, 209).

3. *Plants.* Numerous studies have been carried out on the oxidation of various diamines by plant preparations (82, 142, 143, 205, 279, 446, 448). Recently, using 1,4-diaminobutane as a substrate, the plant enzyme has been purified about 1000-fold (174, 174a, 448). The products are the same as discussed above for animal diamine oxidase. The enzyme is inhibited by cyanide, semicarbazide, diethyldithiocarbamate, and several other metal-binders (174, 278, 279). The purified enzyme contains 0.08 to 0.09% copper. The copper is removed by treatment with diethyldithiocarbamate with concomitant loss of activity; activity is restored by the addition of Cu^{++}. The highly purified enzyme has an absorption maximum at 500 mμ, which disappears on anaerobic incubation with 1,4-diaminobutane, but the responsible prosthetic group has not been identified (279).

4. *Alkaloid biosynthesis: The role of diamines and diamine oxidase.* For many years Schöpf *et al.* (358–361) have been studying the reactions of Δ^1-pyrroline and Δ^1-piperideine as applied to the chemical synthesis of alkaloids. For this reason it was of particular interest to us, when we first obtained evidence that Δ^1-pyrroline and Δ^1-piperideine are formed during the enzymatic oxidation of 1,4-diaminobutane and 1,5-diaminopentane by hog kidney diamine oxidase (412). Subsequently, the formation of Δ^1-pyrroline and Δ^1-piperideine was extensively studied in pea extracts and in plant diamine oxidase preparations by Mann and Smithies (280) and by Hasse and Maisack (159).

Several systems are described below in which C^{14}-1,4-diaminobutane or C^{14}-1,5-diaminopentane is incorporated into various alkaloids that contain piperidine or pyrrolidine rings (fig. 2). Although there is no definitive proof that oxidation by diamine oxidase is the first step in this incorporation, the results, in general, are consistent with this possibility. Various possible formulations have been suggested for the later steps in the biosynthesis of the alkaloids, but direct evidence concerning their validity is not available.

Most of the incorporation experiments have been carried out with intact plants or with isolated shoots, leaves or roots. C^{14}-1,5-diaminopentane is incorporated into lupinine (365, 392), sparteine (364, 365, 367), lupanine, hydroxylupanine (368), matrine (366), and anabasine (253, 301). C^{14}-1,4-diaminobutane is incorporated into nicotine (252). In 1943 Cromwell (83, 84) showed that 1,4-diaminobutane administration increased the yield of hyoscyamine in *Atropa belladonna* and *Datura stramonium*. Although initial experiments with C^{14}-1,4-diaminobutane did not support these findings (93), more recent observations have shown that this diamine is indeed incorporated into the tropine moiety of hyoscyamine (196, 259, 300). There is considerable question, however, as to whether the incorporation of 1,4-diaminobutane into hyoscyamine represents the normal biosynthetic pathway into the alkaloid, since, after C^{14}-ornithine administration,

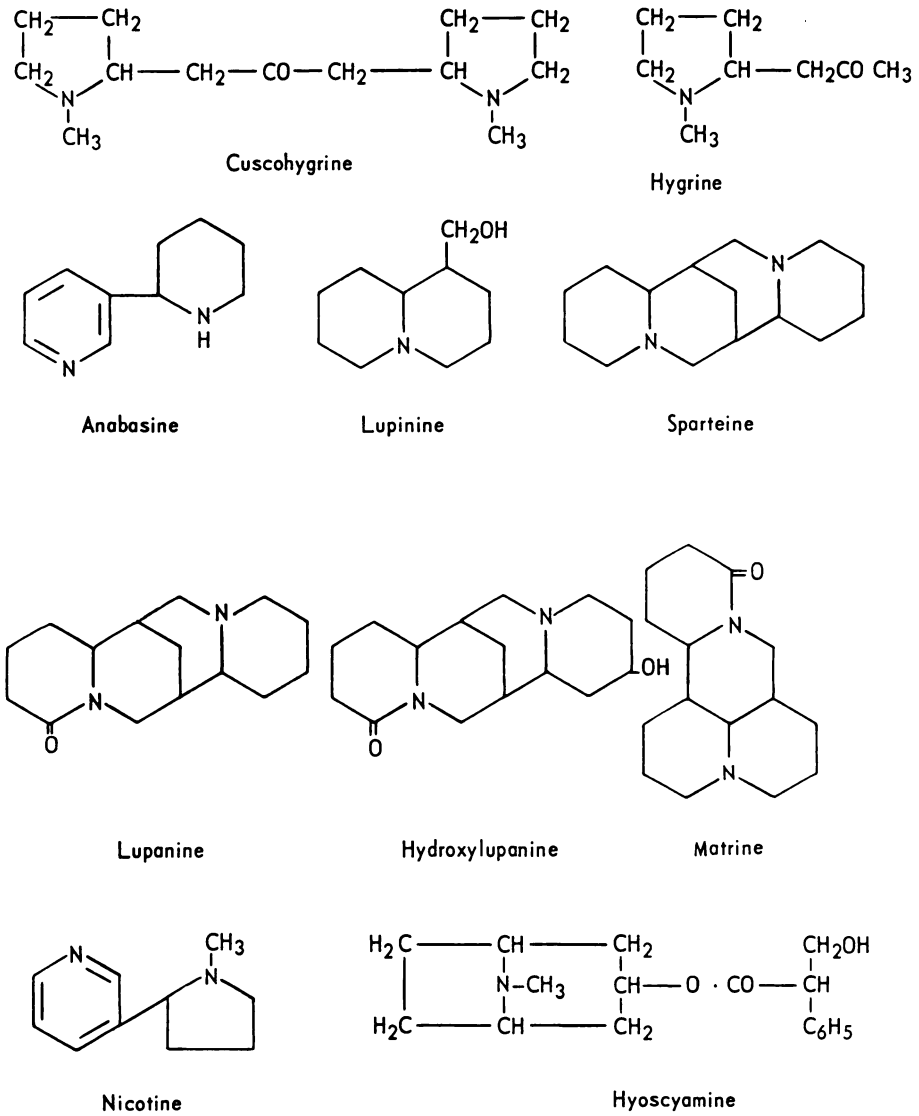


FIG. 2. Structural formulas for alkaloids referred to in text.

the isotope distribution in the alkaloid excludes a symmetrical intermediate (254, 255).

In addition to the above studies with intact plant tissues, synthesis of anabasine has been described in crude pea extracts during the oxidation of 1,5-diaminopentane (158). Similarly hygrine is formed when a diamine oxidase preparation is added to a mixture of N-methyl-1,4-diaminobutane and acetoacetic acid; when acetonedicarboxylic acid is used instead of acetoacetic acid, hygrine and cuscohygrine are formed (434).

C. Nonoxidative metabolism

Acetylation of the polyamines has been observed only in intact bacteria. Normally, only a small amount of monoacetyl-1,4-diaminobutane and monoacetylspermidine occurs in *E. coli* that have been grown on synthetic media. When 1,4-diaminobutane, spermidine, or spermine is added to the medium, the total concentration of the added amine increases in the cell and the percentage of acetyl derivatives present increases markedly (103). When *S. aureus* is grown in a medium containing 1,4-diaminobutane or spermidine, the respective monoacetyl derivatives appear in the medium (341). When spermine is added to the growth medium, the cells contain monoacetylspermine. In addition to the acetyl derivatives, when *E. coli* is grown on spermine or spermidine, a glutathione derivative of each of these amines is found (100, 102).

Transamination. In the previous section (X-B, 2) we have referred to the studies of Kim and Tchen (208, 208a, 209) on a transamination of 1,4-diaminobutane catalyzed by pyridoxal phosphate in extracts of a 1,4-diaminobutane-adapted *E. coli.* Some evidence for a transamination reaction in plant tissues has been presented by Hasse and Schmid (160) with 1,4-diaminobutane, 1,5-diaminopentane, and their acetylated derivatives as donors, and α-ketoglutarate as the acceptor. In the transamination reactions the amines are converted to the same products that are obtained by an oxidative deamination, *i.e.*, the corresponding aminoaldehydes or their cyclic derivatives. Spermidine also serves as a substrate, while the reaction goes very slowly with spermine. With 1,4-diaminobutane and monoacetyl-1,4-diaminobutane, the reaction is reversible. Since the extracts used were contaminated with diamine oxidase, the nature of the reaction is not certain yet, and further work with purified enzymes is required.

Transamidation (*transglutaminase*). Waelsch and co-workers (75, 76, 304, 306, 352) have described a system from guinea pig liver which carries out the incorporation of certain amines into proteins by a transamidation reaction. In this reaction the amine replaces the NH_2 of glutamine residues in the intact protein, and NH_3 is released. The enzyme carrying out this reaction is stimulated by Ca^{++}. Many amines will serve as the substrate, including 1,4-diaminobutane, 1,5-diaminopentane, spermidine, and spermine. A wide variety of proteins will act as acceptors, *e.g.*, insulin, fibrinogen, casein, pepsin, β-lactoglobulin, and α-globulin, as well as crude tissue homogenates.

Carbamylation of 1,4-diaminobutane has been reported with extracts of bakers' yeast and of *A. nidulans* pu_1 (133). A *transamidination* reaction has been postulated for the incorporation of the guanido groups of arginine into hirudonine in leeches (335).

XI. CHEMICAL STUDIES

Chemical syntheses have been published for the preparation of nonisotopic and isotopic (C^{14}, H^3, N^{15}) diamines (14, 75, 93, 110, 121, 145, 188, 253, 301, 317, 369, 425),[7] spermidine, spermine, and related compounds (56, 75, 85, 189, 190, 214,

[7] A more complete list of references to syntheses of various diamines may be found in Beilstein's Handbuch der organischen Chemie, vol. IV, system No. 344.

363, 409, 426). The most convenient method for the synthesis of spermidine and spermine depends on the condensation of acrylonitrile with 1,4-diaminobutane, and reduction of the corresponding nitrile:

(1) $NH_2(CH_2)_4NH_2 + CH_2{=}CHCN \rightarrow NH_2(CH_2)_4NHCH_2CH_2CN$
$NH_2(CH_2)_4NH_2 + 2\ CH_2{=}CHCN \rightarrow NCCH_2CH_2NH(CH_2)_4NHCH_2CH_2CN$

(2) $NH_2(CH_2)_4NHCH_2CH_2CN \xrightarrow{H_2} NH_2(CH_2)_4NHCH_2CH_2CH_2NH_2$
$NCCH_2CH_2NH(CH_2)_4NHCH_2CH_2CN \xrightarrow{H_2} NH_2CH_2CH_2CH_2NH(CH_2)_4NHCH_2CH_2CH_2NH_2$

Tritiated spermidine and spermine with very high specific activities are made commercially by the Wilzbach procedure (454) followed by chromatography of the crude product on Dowex 50.

Labeled spermidine and spermine can also be prepared biosynthetically by adding isotopic 1,4-diaminobutane to growing cultures of the 1,4-diaminobutane-requiring mutant of *A. nidulans* (419) or by adding labeled methionine to the methionine-requiring mutant of this organism (26) or of *N. crassa* (144).

Synthetic procedures have also been developed for the monoacetyl derivatives of 1,4-diaminobutane, 1,5-diaminopentane, spermidine (both isomers[4]), and spermine (32, 103, 161, 189, 409).

The ionization of various diamines and polyamines has been studied by Rometsch *et al.* (338) and by Schwarzenbach (370), although no values are reported for spermidine or spermine. The dissociation constants (pK) for 1,4-diaminobutane are 10.4 and 9.3; for 1,5-diaminopentane, 11.0 and 9.7 (338).

Evidence for the formation of *complexes* between metal ions and polyamines has been presented by several laboratories (38, 42, 154, 282, 308). In general, the strength of these complexes is greatest for 1,2-diaminoethane, 1,3-diaminopropane, and for compounds containing these groups. In compounds with greater distances between the nitrogen groups (as 1,4-diaminobutane and 1,5-diaminopentane) the tendency to form complexes is markedly diminished since chelate rings containing greater than six atoms would be relatively unstable (42, 282). Spermidine and spermine were not included, however, in the above studies.

Evidence for complex formation between spermine and citrate has been obtained by measuring the change in the titration curve obtained by the addition of spermine hydrochloride to solutions of citric acid (416). Spermine and spermidine have a high affinity for acidic ion-exchange resins (343) and displace less basic compounds; it has been suggested (245, 246) that analogous ion-exchange phenomena occurring in biological systems may explain the biological activity of amines.

Infrared spectra. Infrared spectra have been reported for spermine (349), spermidine (206, 349), and for a series of diamines (206, 210, 349, 371). *Proton magnetic resonance* studies have been reported for spermidine (315). *X-Ray powder diffraction* diagrams for several diamines have been presented by Brock and

Hannum (60). The crystal structure of spermine phosphate hexahydrate has recently been described by Iitaka and Huse (187a).

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The interest of this laboratory in the polyamine field began with the studies of Dr. Sanford Rosenthal on the renal toxicity of spermine. We wish to take this opportunity of acknowledging the collaboration and advice of Dr. Rosenthal during our subsequent studies.

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