ASPOROGENOUS MUTANT OF *BACILLUS SUBTILIS*

BIOCHEMICAL INVESTIGATIONS ON AN ASPOROGENOUS MUTANT OF *BACILLUS SUBTILIS*

By

CHARLES TAI-CHIEN CHOW, B.Sc., M.Sc.

A Thesis

Submitted to the Faculty of Graduate Studies

in Partial Fulfilment of the Requirements

for the Degree

Doctor of Philosophy

McMaster University

January 1971

DOCTOR OF PHILOSOPHY (1971)
(Biology)

McMASTER UNIVERSITY
Hamilton, Ontario

TITLE: Biochemical investigations on an asporogenous mutant of *Bacillus subtilis*

AUTHOR: Charles Tai-Chien Chow, B.Sc. (National Taiwan University, Taiwan, China)
M.Sc. (University of Alberta)

SUPERVISOR: Professor I. Takahashi

NUMBER OF PAGES: vii,93

SCOPE AND CONTENTS:

Deletion in the chromosome of *Bacillus subtilis* strain $Sp^{-}H12-3$ was demonstrated by Hg-Cs_2SO_4 density gradient centrifugation. The base composition of the deleted DNA segments and transcription of m-RNA from these DNA segments were investigated. Physiological and biochemical studies of the mutant $Sp^{-}H12-3$ yielded information on uridine derivatives which may be intimately associated with the process of sporulation.

PREFACE

This thesis describes studies carried out in the Department of Biology, McMaster University, from July 1968 to September 1970. Except where specifically mentioned, it consists entirely of my own original work. No similar thesis has been submitted to any other university.

I would like to express my sincere gratitude to Dr. I. Takahashi for his invaluable guidance, encouragement and criticisms throughout the work. Thanks are also due to Drs. H. Yamagishi and T. Morimoto for their assistance on many occasions, and to the National Research Council of Canada for the financial support during the period of research. I would also like to thank my typist, Mrs. Mary Haight, for her skillful and efficient typing.

Department of Biology,
McMaster University,
Hamilton, Ontario, Canada.
January, 1971.

TABLE OF CONTENTS

Chapter		Page
I	Introduction	1
II	Materials and Methods	12
III	Deletion in Strain $Sp^{-}H12-3$	34
IV	Physiological and biochemical changes in strain $Sp^{-}H12-3$	59
V	Discussion	78
	Bibliography	82

LIST OF FIGURES

Figure		Page
1	Incorporation and chasing of ^{3}H-uridine in *B. subtilis* strains A26, $Sp^{-}H12$-3 and $Sp^{-}N2$-2 in the log-phase	36
2	Incorporation and chasing of ^{3}H-uridine in *B. subtilis* strains A26, $Sp^{-}H12$-3 and $Sp^{-}N2$-2 at t_0	37
3	Incorporation and chasing of ^{3}H-uridine in *B. subtilis* strains A26, $Sp^{-}H12$-3 and $Sp^{-}N2$-2 at t_1	38
4	Incorporation and chasing of ^{3}H-uridine in *B. subtilis* strains A26, $Sp^{-}H12$-3 and $Sp^{-}N2$-2 at t_2	39
5	Incorporation and chasing of ^{3}H-uridine in *B. subtilis* strains A26, $Sp^{-}H12$-3 and $Sp^{-}N2$-2 at t_3	40
6	Sedimentation profile of ^{3}H-labelled A26 t_2 RNA in a sucrose gradient	42
7	Enrichment of m-RNA by absorption on $Sp^{-}H12$-3 DNA	43
8	Competition between ^{3}H-A26 t_2 RNA and unlabelled A26 or $Sp^{-}H12$-3 t_2 RNA on DNA sites	46
9	Competition between the "$Sp^{-}H12$-3 treated" or "A26 treated" ^{3}H-A26 t_2 Gr. II RNA and unlabelled A26 or $Sp^{-}H12$-3 t_2 RNA on A26 DNA sites	48
10	Distribution of ^{3}H-A26 and ^{14}C-$Sp^{-}H12$-3 DNA in a CsCl density gradient	51
11	Distribution of ^{3}H-A26 and ^{14}C-$Sp^{-}H12$-3 DNA fragments in a Hg-Cs_2SO_4 density gradient	53
12	Analysis of fractionated DNA fragments by DNA-DNA hybridization	55

Figure		Page
13	Distribution of unlabelled A26 and Sp$^-$H12-3 DNA in a Hg-Cs_2SO_4 density gradient	57
14	Hybridization between fractionated DNA fragments and pulse-labelled A26 t_2 RNA	58
15	Changes in pH and pyruvic acid in A26 and Sp$^-$H12-3	61
16	Elution profiles of TCA-soluble nucleotides extracted from A26 and Sp$^-$H12-3 in the log-phase	63
17	Elution profiles of TCA-soluble nucleotides extracted from A26 and Sp$^-$H12-3 at t_0	64
18	Elution profiles of TCA-soluble nucleotides extracted from A26 and Sp$^-$H12-3 at t_1	65
19	Elution profiles of TCA-soluble nucleotides extracted from A26 and Sp$^-$H12-3 at t_2	66
20	Elution profiles of TCA-soluble nucleotides extracted from A26 and Sp$^-$H12-3 at t_3	67
21	Relative positions of some uridine-containing nucleotides and their derivatives	68
22	Absorption spectrum of N-acetylglucosamine and the sugar moiety isolated from nucleotide X_1 after reaction with dimethylaminobenzaldehyde	69
23	Changes in nucleic acids and TCA-soluble nucleotides in strains A26 and Sp$^-$H12-3 at early stages of sporulation	74
24	Activity of UDP-gal-4-epimerase in A26, Sp$^-$H12-3 and Sp$^-$N2-2	76
25	Activity of galactose-1-phosphate uridyl transferase in A26, Sp$^-$H12-3 and Sp$^-$N2-2	77

LIST OF TABLES

Table		Page
I	Uridine, phosphorous and hexose contents in nucleotides X_1, X_2 and X_3	71
II	Acid-soluble nucleotides in A26 and $Sp^-H1$2-3	72

CHAPTER I

INTRODUCTION

Bacterial sporogenesis has been extensively investigated since the end of the last century (51,85, 96) and shown to be useful in the study of cellular differentiation. Information obtained in such a system should be helpful to the analysis of developmental problems in higher organisms. The application of advanced techniques and equipment (e.g., electron microscope, radioactive isotopes, ultracentrifuges, etc.) to the study of sporogenesis has led to a clearer understanding of spore formation (61,139,140). Recent publications dealing with bacterial sporulation have been reviewed by a number of authors (32,52,72,103,126). The scope of the present review will be limited to information concerning sporogenesis of *Bacillus* species.

Successive changes in cell structure which occur during sporulation have been first described in *B. cereus* by Young and Fitz-James (139,140). Observations made with other spore-forming species (11,34,94,97) are generally very similar to those of the above authors. Vegetative cells are converted to spores in a relatively synchronous fashion through an intricate sequence of processes.

These changes are continuous, but it is convenient to select a few typical stages. The time when the exponential growth of cells ends is arbitrarily taken as t_0 of sporulation, and x hours after that is t_x (103). Cells of *B. subtilis* are said to be at stage I (t_0 - t_1) when the chromatin is visible as a single axial rod, rather than the one or two nuclear bodies observed in the exponentially growing cells (98). Stage II (t_1 - t_2) begins with an invagination of the cytoplasmic membrane and ends with the completion of a spore septum, dividing the nuclear material in two, and creating a double-celled organism. At stage III (t_2 - t_3), further elongation of the double membrane, which bulges into the bigger part of the cell, is accompanied by a polewards displacement of the insertion point of the septum, until an entirely intracytoplasmic forespore is "carved out" of the mother cell. The next two stages are characterized by the formation of new envelopes around the forespore. The cortex is formed between the two membranes at stage IV (t_3 - t_4), and the two layers of the spore coat, exterior to the cortex, begin to be formed at stage V (t_4 - $t_{5.5}$). Towards the end of stage V and during stage VI ($t_{5.5}$ - t_7), the spore undergoes a number of changes which have been described as maturation. The spore becomes heat-resistant and its refractility increases. Lysis of the mother cell eventually sets the mature spore free, and this occurs in the last stage (VII, after t_7).

In concert with the cytological changes mentioned above, a number of biochemical changes take place during sporulation (32,52,103,113,139,140). One of the changes occurs at the transcription level. Young and Fitz-James (139,140) have shown that when *B. cereus* cells previously labelled with ^{32}P are placed in a non-radioactive medium at t_0 - t_2, the specific radioactivity of the labelled RNA decreases rapidly. This observation suggests that a turnover of certain RNA species takes place during sporulation. Similar evidence has been obtained in *B. subtilis* by Spotts and Szulmajster (113) and by Balassa (8). Using the DNA-RNA hybrid competition technique, Doi (26) has reported that some of the messenger-RNA (m-RNA) made in *B. subtilis* cells during sporulation are specific for sporulation. Similar observations have been made with *B. cereus* by Aronson (4) and with *B. subtilis* by Yamagishi and Takahashi (136). Szulmajster *et al.* (118) have shown that addition of actinomycin D to *B. subtilis* cultures in early stages of sporulation (before t_3) inhibits the formation of thermoresistant spores. These results lead the above authors to propose that some of the sporulation m-RNA are probably synthesized during that period (118). However, other species of m-RNA besides the sporulation m-RNA are also being produced at the same time (4,7,8,26,113,136).

It has been demonstrated in many cases that studies on m-RNA for specific functions will complement

with biochemical and genetic aspects of investigations. Takahashi (120,123,124) has shown that during the early stages of sporulation (from t_0 to t_2), the mutant Sp^-N2-2 is able to carry out some functions related to sporulation (e.g., formation of septum, and production of extracellular proteases and antibiotics). This mutant is therefore thought to be blocked at a late stage of sporulation (122,124 and Takahashi's unpublished data). On the other hand, the mutant Sp^-H12-3 does not show any of the sporulation-associated events, whether early or late (122,124, and Takahashi's unpublished data). Genetic analyses carried out by Takahashi (122,123,124) on Sp^-H12-3 have indicated that this strain is probably a deletion mutant. This interpretation has been further strengthened by the results of DNA-RNA hybrid competition experiments carried out by Yamagishi and Takahashi (136). They have shown that when wild-type DNA and ^3H-RNA extracted from sporulating cells at t_1 are hybridized in the presence of a large amount of unlabelled RNA from either wild-type or the mutant strain, the competing capacity of unlabelled RNA extracted at t_1 from wild-type cells is higher than that from Sp^-H12-3. This difference in competing capacity disappears when DNA from Sp^-H12-3 is used instead of the wild-type DNA. These results lead the above authors to conclude that a population of m-RNA is not produced in Sp^-H12-3 at t_1 because of the deletion in its chromosome (136). In the same study (136),

Sp^-N2-2 has been shown to be able to produce this population of m-RNA at t_1. At t_3, however, some of m-RNA which are being made in wild-type cells, are found to be absent in this mutant.

It has been reported that an active turnover of protein takes place during sporulation (8,113,140). Spotts and Szulmajster (113) have demonstrated that the rate of protein synthesis during stationary phase in a sporulating strain of *B. subtilis* is from 2 to 10 times higher than that in an asporogenous mutant. A number of enzyme activities have been found to appear or increase significantly during sporulation (32,52).

Young and Fitz-James (139) have reported that when *B. cereus* cells previously labelled with ^{32}P are transferred into a non-radioactive medium at t_1, there is no difference between the specific activity of the DNA-phosphorus in the cells and in the spores formed following transfer. On the other hand, when this transfer is made at, or before, t_0, the specific activity of the DNA-P in the spores is lower than that in the progenitor cells. From these results, they have suggested that DNA which will be later incorporated into spores is already present in the cells at t_1. Canfield and Szulmajster (19), by the ^{32}P-suicide technique, have shown that DNA which enters into spores of *B. subtilis* is produced before t_2.

Culture filtrates of *Bacillus* species show usually strong proteolytic activities (23,89,132). Studies on the protease production in *B. larvae* (37), *B. licheniformis* (15), *B. subtilis* (22,68,70), and *B. cereus* (58) have shown that a close correlation exists between the production of protease and sporulation. The protease activity increases rapidly at the end of vegetative growth, and this increase is a result of *de novo* synthesis, rather than delayed excretion or activation of preformed enzymes (15,22,58,68).

Two RNA-degrading enzymes have been isolated and characterized in culture filtrates of *B. subtilis*. They are a neutral ribonuclease (RNase), appearing at the end of exponential growth (22,64,78) and a phosphodiesterase which also degrades DNA, appears shortly after t_0 (48,73,83).

Antibiotic activities against *Staphylococcus aureus* (1,10,103) or *B. subtilis* strain H (112) are detectable at an early stage of sporulation in Marburg strain of *B. subtilis*. Genetic analyses of asporogenous ($\underline{sp}^-$) mutants show that these two antibiotics are non-identical (9). A close correlation between the antibiotic production and sporulation has been suggested by Schaeffer *et al.* (103). These authors have shown that sporulation mutants which are blocked at the first stage of development produce no antibiotic activities, whereas mutants blocked at a later stage are able to produce the antibiotics.

Other biochemical events associated with sporulation are the formation of wall-lytic enzymes (30,79,80,92,93,115, 137), amylase (89) and lethal toxins (12,43,51,71,114,140) all of which appear at late stages of sporulation.

While, as described earlier, a number of products (enzymes, antibiotics, etc.) are considered, by many authors, to be intimately related to the process of sporulation, thus far only one product, dipicolinic acid (DPA), has been found to be specifically related to the process of sporulation (133). Using a Renografin-76 density gradient, Wise *et al.* (133) have succeeded in obtaining spores of DPA-less mutants of *B. cereus* which lose viability completely within one to two minutes at 80°C. In contrast, the wild-type spores show no drop in viability at this temperature for 30 minutes. When DPA is added to the mutant cultures, the resulting spores are completely heat resistant. Studies carried out with ^{14}C-labelled DPA indicate that the added DPA is actually incorporated into these spores.

Physiological changes in sporulating cells have been studied extensively and reviewed by a number of authors (32,33,40,78,100). During the exponential growth phase, the carbon source, such as glucose, in growth medium is converted to organic acids (pyruvate and acetate), resulting in a decrease in pH value of the culture fluid (32,78). After t_0, organic acids are re-utilized and the pH rises. Studies on the metabolism of organic acids

in sporulating cells have led Halvorson (32) to postulate that these acids are metabolized via the glyoxylic acid cycle. Later studies on this aspect (27,119) have shown that mutant strains of *B. subtilis* which are unable to produce enzymes for the glyoxylic acid cycle are asporogenous. The changes in pH as well as production and re-utilization of pyruvic acid have been chosen as two parameters for physiological changes and investigated with a wild-type culture and Sp^{-}H12-3 in the present study.

In order to facilitate biochemical investigations and to understand control mechanism of sporulation, several laboratories have initiated work on genetic aspects of sporulation. Asporogenous mutants of *B. subtilis* have been isolated and analysed by transformation (101,111,112,121,123) and by transduction (120,123). When grown on Schaeffer's sporulation agar, mutant strains form colonies which lack dark brown pigment, and therefore can be distinguished readily from the wild-type colonies which are pigmented (6,11,40,100,101). Takahashi (124) has proposed to divide $\underline{sp^{-}}$ mutants of *B. subtilis* into three classes according to their frequencies of sporogenous ($\underline{sp^{+}}$) revertants. Class I mutants show a reversion frequency of less than 10^{-10}; Class II mutants, which are presumably point mutants, produce $\underline{sp^{+}}$ revertants at a frequency of 10^{-7} to 10^{-10}; and Class III (oligosporogenous) mutants have a frequency higher than that of Class II mutants. Some spore-forming

albino mutants have been described in *B. subtilis* (101,102). They can be distinguished readily from true sp⁻ mutants by direct microscopic examinations.

Spizizen (112) has reported a class of *B. subtilis* sp⁻ mutants with a genetic defect in one locus which is clustered with several genes controlling the production of two proteolytic enzymes, an antibacterial substance and a competence factor. Schaeffer and Ionesco (101), by the transformation technique, have observed three unlinked loci which control sporulation of *B. subtilis* with no associated auxotrophic markers. Takahashi (120,121), on the other hand, has reported that sporulation mutations can occur at scores of unlinked genes on the chromosome of *B. subtilis*. By the use of an indirect mapping technique with transformation and transduction, Takahashi (120,121) has succeeded in establishing the chromosomal location of some spore genes in *B. subtilis*.

The control mechanism for sporulation is still unknown. Two models have been postulated by Halvorson (33). The first model postulates that the order of the spore genes on the chromosome corresponds to the sequence of biochemical changes occurring in sporulation, and that transcription of the spore genes is unidirectional. The second model postulates a sequential induction of the spore genes. According to this model, the order of biochemical changes is determined because the product of

the first enzyme acts as both inducer and also as substrate of the second enzyme. Hoch and Spizizen (36), by studying a number of spo⁻ mutants of *B. subtilis*, have shown that mutations leading to the complete loss of all sporulation-associated biochemical functions map in a tight cluster in the terminal region of *B. subtilis* chromosome. From their genetic data, they have suggested that the early events in sporulation are under positive control, and that control is mediated, at least in part, by the product of the locus (spo A) in the terminal region. Another interesting control mechanism of sporulation has been recently suggested by Losick and Sonenshein (59). They postulate that during sporulation a protein molecule may act as a positive control element by associating with RNA polymerase. This association may change the template specificity of the enzyme and initiate the transcription of sporulation genes which are not expressed during the vegetative growth.

A number of nucleotides have been shown to play a role in the regulation of enzyme synthesis, e.g., dTTP for thymidine kinase (16,42,82), dCTP for CDP reductase (76,77), ATP and dTTP in reduction of CDP and UDP (55), GTP and 3',5'-cyclic GMP in translation of protein (53,54), etc. Perhaps, the most interesting among the nucleotides having such properties is 3',5'-cyclic AMP which acts as a positive regulator in multicellular organisms and in

E. coli (62,84,86,88,90,117). It has been shown by many authors that 3',5'-cyclic AMP stimulates the synthesis of β-galactosidase (62,86,87) and overcomes catabolite or transient repression of β-galactosidase caused by glucose in *E. coli* cells (29,62,84,86,87,88). Both functions (stimulation of enzyme synthesis and "derepression" of β-galactosidase) of 3',5'-cyclic AMP are thought to occur at the level of transcription (86,88). Since it appears that during vegetative growth genes controlling sporulation are non-functional, but are expressed during sporulation, the expression of these genes also may be under the control of small molecules such as nucleotides. In order to test this possibility, acid-soluble nucleotides in wild-type cells and Sp^-H12-3 are thoroughly investigated in the present study.

CHAPTER II

MATERIALS AND METHODS

1. Symbols

Symbols to designate the genotype of mutant bacteria are according to Demerec *et al.*(25).

The abbreviations used for amino acids, bases, nucleosides, nucleotides and nucleic acids are those recommended by the NAS-NRC Office of Biochemical Nomenclature (41).

2. Bacterial Strains

Strains of *Bacillus subtilis* used are listed below (121,122,123,124):

Strain	Origin	Characteristics
SB19E	SB19 (E. W. Nester)	sp^+, str^r, ery^r, prototrophic
A26	W (C. Anagnostopoulos)	sp^+, ura
Sp^-H12-3	H12 (I. Takahashi)	sp^-, phe
Sp^-N2-2	N2 (I. Takahashi)	sp^-, ser

3. Culture media

a) Difco Penassay Broth

3. b) Difco Tryptose Blood Agar Base (TBB agar)

c) Spizizen's Minimal Medium (SMM) (111)

$(NH_4)_2SO_4$	2.0 g
K_2HPO_4	14.0 g
KH_2PO_4	6.0 g
Sodium citrate ($Na_3C_6H_5O_7.2H_2O$)	1.0 g
$MgSO_4.7H_2O$	0.2 g
Distilled water	1000.0 ml

The pH was adjusted to 7.0. Before use, 50 ml of sterile 10% glucose solution were added to every litre of the medium. The minimal agar contained 15 g of Bacto-agar per litre. Where biochemical supplements were required in the medium, they were autoclaved separately and added at a final concentration of 25 µg/ml for phenylalanine or 10 µg/ml for uracil.

d) Schaeffer's Sporulation Medium (SSM) (99)

Nutrient broth (Difco)	8.0 g
$MgSO_4.7H_2O$	0.25 g
KCl	1.0 g
$MnCl_2$ (1.9% solution)	0.1 ml
Distilled water	1000.0 ml

The pH was adjusted to 7.0. Before use, the following sterile solutions were added:

$FeSO_4.7H_2O$ (0.27%)	0.1 ml
$Ca(NO_3)_2.4H_2O$ (2.3%)	10.0 ml

Sporulation agar contained 15 g of Bacto-agar per litre.

3. e) Tris-medium

Yeast extract	0.5 g
Casamino acids	0.5 g
$(NH_4)_2SO_4$	2.0 g
Sodium citrate	1.0 g
$MgSO_4.7H_2O$	0.2 g
Tris-HCl buffer (1 M, pH 7.5)	50.0 ml
Potassium phosphate buffer (0.05 M, pH 7.5)	2.0 ml
Distilled water	1000.0 ml

After autoclaving, the following sterile solutions were added:

$FeCl_3$ (0.5%)	0.5 ml
Glucose (10%)	50.0 ml

f) Preserving medium for asporogenous strains

Penassay Broth (Difco) containing 15% glycerol.

4. Chemicals

All the chemical compounds used were analytical grade. Amino acids were purchased from Calbiochem Co. Bases, nucleosides and nucleotides were products of Sigma Chemical Co., and Nutritional Biochemical Co., Ltd. Calf thymus DNA and yeast RNA were products of Worthington Biochemical Co. Albumin (Bovine Fraction V) was obtained from Calbiochem. Co. 3,5-Diaminobenzoic acid was purchased from Aldrich Chemical Co. Caesium chloride purchased from

Harshaw Chemical and caesium sulphate obtained from Kawecki Chemical Co. were both of optical grade.

All ^{3}H and ^{14}C labelled compounds were purchased from New England Nuclear Co. Orthophosphate ($H_3{}^{32}PO_4$) was obtained from Atomic Energy of Canada Ltd.

5. Enzymes

Lysozyme (egg white), pronase, glucose-6-phosphate dehydrogenase, phosphoglucomutase and UDPG dehydrogenase were purchased from Calbiochem Co. Deoxyribonuclease (pancreatic) and ribonuclease (pancreatic) were products of Worthington Biochemical Co.

6. Other materials and solutions

Dowex-1, X-8 (200-400 mesh) was purchased from Bio Rad Laboratories. Nitrocellulose membrane filter (Type B-6) and S & S analytical filter paper disks (No. 589) were products of Schleicher and Schuell, Inc. Liquifluor (25 X concentration liquid scintillator) was purchased from Nuclear Chicago Inc.

Scintillation fluid was a mixture of Liquifluor (100 ml) and toluene (2370 ml). This mixture contained 4 g of PPO and 50 mg of POPOP in every litre. Saline sodium citrate (SSC) was prepared by dissolving 8.5 g of NaCl and 4.4 g of sodium citrate in 1000 ml of distilled water. The pH was adjusted to 7.0.

7. Maintenance of bacterial stocks

Sporogenous strains of *B. subtilis* were kept as spores on SSM agar slants at 4°C. When required, spores of stock cultures were streaked on TBB agar and incubated at 37°C overnight.

Asporogenous strains were kept frozen at -40°C in the preserving medium.

8. Bacterial culture

Broth cultures were obtained by inoculating a medium with cells grown on TBB agar overnight, and incubated at 37°C for 4 to 5 hours in a water-bath shaker. The growth of bacteria in broth was determined by means of a Klett-Summerson colorimeter equipped with filter No. 54 (wavelength range, 500-700 nm). Viable cell counts were determined, after appropriate dilutions in SMM, by plating 0.1 ml samples on TBB agar and incubating at 37°C overnight. The number of spores was determined by heating the cell suspension at 85°C for 10 minutes and plating on SSM agar plates.

9. Pyruvic acid

The amount of pyruvic acid was determined by the method of Nakata and Halvorson (74). Cells were grown in SSM and at intervals 3 ml samples were transferred into tubes containing an equal volume of cold 10% trichloroacetic

acid (TCA), mixed with a Vortex for a few seconds, and kept in the cold for at least 60 minutes. The TCA-treated cells were centrifuged at 7,000 x g for 10 minutes in a Sorvall Model RC-2 centrifuge. The supernatant fluid obtained was warmed to room temperature. One ml of 2,4-dinitrophenylhydrazine solution (1 mg/ml in 2 N HCl) was added to 3 ml of the warmed supernatant fluid. After the reaction mixture was stirred with a Vortex for 5 minutes, 8 ml of toluene were added and stirred further for 20 seconds. The aqueous layer was removed by a pipette and discarded. To the upper solvent layer, 6 ml of 10% Na_2CO_3 solution were added and the two phases were mixed. The lower aqueous layer was carefully removed into another tube containing 5 ml of 1.5 N NaOH. The mixture was stirred with a Vortex for 1 minute, and 10 minutes later its optical density was measured at 440 nm in a Beckman Spectrophotometer Model DU. Potassium pyruvate was used to construct a standard curve.

10. Preparation of DNA

The technique described by Takahashi (121) was used to prepare crude DNA. Crude DNA solution in 1 X SSC was treated with boiled pancreatic RNase (10 µg/ml) for 2 hours at 37°C, and then with self-digested pronase (50 µg/ml) for 2 hours at 37°C. The enzyme-treated DNA preparation was deproteinized by the sodium lauryl sulfate method (121) and

finally purified by the isopropanol precipitation technique described by Marmur (66).

^{3}H- or ^{14}C-thymidine labelled DNA was prepared as follows: cells were grown in SMM containing Casamino Acids (0.02%), 5'-dAMP (1.5×10^{-3} M), and phenylalanine at 25 µg/ml for strain $Sp^{-}H12$-3 or uracil at 10 µg/ml for strain A26, for 3 hours in a 37°C water-bath shaker. The precultures were diluted in the above medium containing 10 µc of ^{3}H-thymidine (sp. act. = 15 C/mM), or 0.5 µc of ^{14}C-thymidine (sp. act. = 50 mC/mM) per ml. Incubation continued for 4 hours and labelled DNA was extracted and purified as described above. The radioactivity of the labelled DNA was determined by precipitating an aliquot of DNA solution in 10 ml of cold 5% TCA and then filtered through a nitrocellulose membrane filter (S & S, No. B-6) which was washed with 50 ml of 5% TCA and dried under an infrared lamp. The dried membrane filter was then put into a vial containing 10 ml of scintillation fluid and counted in a Beckman Model LS-250 liquid scintillation counter.

The amount of DNA was estimated by the diphenylamine method of Burton (17) or by measuring the optical density at 260 nm, assuming $E_{1\ cm}^{0.1\%} = 20.0$. [redacted] only a minute amount of DNA was available for analysis, the following fluorometric method was employed (49). A dark brown solution of 3,5-diaminobenzoic acid (0.6 g in 2 ml of 4 N HCl) was decolourized by treatment with acid-washed active charcoal

(0.02 g/ml solution). The decolourization was repeated 5 times. Samples of DNA (0.02 ml) in 0.1 M NaCl were mixed with 0.03 ml of 1.8 N perchloric acid (PCA) and 0.05 ml of the decolourized diaminobenzoic acid solution. The reaction mixture was incubated at 60°C for 30 minutes, and then 1 ml of 0.6 N PCA was added to the mixture. The fluorescence thus formed was measured in a Farrand Fluorometer Model A-2 using primary (Corning filters 5970, 4308 and 3060) and secondary (Corning filters 5031 and 3384) filters. Under these conditions, with calf thymus DNA as standard, a linear relation was obtained between the amount of fluorescence produced and the concentration of DNA ranging from 1.0 to 50 µg per tube.

The method for isolation of DNA from phage T4 was described by Mandell and Hershey (63).

11. Preparation of RNA

Cells of strain A26 were grown in SSM and pulse-labelled with ^{3}H-uridine (sp. act. = 28 C/mM). The cultures in the log-phase were labelled at 10 µc/ml for 1 minute and those of later stages were labelled at 20 µc/ml for 3 minutes. The labelled culture was poured into an equal volume of precooled Tris buffer containing 0.02 M Tris, 0.01 M $MgCl_2$ and 0.02 M NaN_3, pH 7.3. Cells were collected by centrifugation, disrupted by grinding with quartz sand, and resuspended in 5 ml of the Tris

buffer containing DNase at 20 µg/ml. The cell debris and sand were removed by centrifugation at 7,000 x g for 10 minutes. To the supernatant fluid, 0.15 ml of 10% sodium lauryl sulfate solution and 5 drops of 0.02 M acetic acid were added to bring the pH to 5.2. The mixture was shaken with an equal volume of phenol equilibrated with a Tris buffer (0.02 M Tris, 0.01 M $MgCl_2$ and 0.02 M NaN_3, pH 7.3). The two phases were separated by centrifugation (5,000 x g for 5 minutes), and the aqueous layer was carefully pipetted into a tube containing 2 volumes of cold 95% ethanol, mixed well and stored at -40°C overnight. The RNA precipitate obtained was redissolved in an acetate buffer (0.02 M sodium acetate, 0.02 M KCl and 10 mM $MgCl_2$, pH 5.2) and then reprecipitated with ethanol. The last step was repeated three times and the final precipitate was dissolved in 0.2 to 0.3 ml of the acetate buffer. The purity of RNA was checked by measuring its optical densities at 230,260 and 280 nm. The degree of contamination by DNA was found to be less than 1% as determined by the fluorometric method. The radioactivity of labelled RNA was measured by the method described in section 10. The concentration of RNA was determined by either the orcinol method (69) or from the optical density measured at 260 nm, taking $E^{0.1\%}_{1\ cm} = 25.0$. Unlabelled RNA was also prepared by the above technique.

12. Fractionation of labelled RNA

Two-tenths ml of labelled RNA in acetate buffer were layered on top of a linear sucrose gradient (4.7 ml, 15-30%) and centrifuged at 35,000 r.p.m. for 15 hours in an SW 50 rotor at 4°C in a Beckman L2-65 ultracentrifuge. After centrifugation, the bottom of the tube was punctured and 4-drop fractions were collected. The radioactivity and optical density of each fraction were determined by the methods described earlier.

13. DNA-DNA hybridization

The method described by Warnaar and Cohen was employed in the study of DNA-DNA hybridization (128). The DNA was immobilized on nitrocellulose membrane filters (S & S, No. B-6) according to the method of Gillespie and Spiegelman (28). The efficiency of immobilization (DNA immobilized/input DNA) was found to be 0.96 to 0.99 when 10 µg, or more, DNA were fixed. Samples of ^{3}H- or ^{14}C-labelled DNA in 0.05 x SSC were sheared by sonication in a Biosonic Model III at step 3 for 30 seconds. Sheared DNA was denatured by heating in a boiling water-bath for 15 minutes and cooled rapidly in an ice-water bath. Hybridization between labelled DNA and unlabelled DNA immobilized on nitrocellulose membranes was carried out in 2 x SSC containing 0.01 M Tris, pH 7.0 at 66°C for 24 hours. After hybridization, membranes were washed with 100 ml

(50 ml for each side of the membranes) of 0.1 x SSC containing 3 x 10^{-3} M Tris, pH 9.4, dried and counted for radioactivities.

14. DNA-RNA Hybridization

The technique of Gillespie and Spiegelman (28) was employed to carry out the hybridization experiments. Annealing mixture contained 1.0 ml of 6 x SSC containing 0.1% sodium lauryl sulfate, various amounts of labelled RNA and a nitrocellulose membrane on which DNA was fixed. The final volume of the annealing mixture was 1.1 ml. For hybrid competition experiments, various amounts of unlabelled RNA was included in the above mixtures. After incubation at 66°C for 16 hours, the annealing mixture was cooled rapidly in an ice-water bath. The membrane was removed and rinsed in 2 x SSC, followed by treatment with boiled RNase at 20 μg/ml for 1 hour at room temperature. It was then washed in 100 ml of 2 x SSC with suction, and the radioactivity determined.

When hybridization was carried out with DNA fractions obtained from a Hg-Cs_2SO_4 density gradient centrifugation, the technique of Nygaard and Hall modified by Yamagishi and Takahashi (136) was used.

15. Nucleotide analysis of DNA

To obtain ^{32}P-labelled DNA, strain A26 was grown in

Tris medium containing 5 μc $H_3{}^{32}PO_4$ per ml. Teichoic acid still present in purified DNA preparations was removed by passing through an agarose column (138). For nucleotide analysis, ^{32}P-labelled DNA was hydrolyzed to nucleoside-5'-monophosphates according to the method of Wu and Kaiser (134) and subjected to paper chromatography (65).

16. Density gradient centrifugation of DNA

a) CsCl density centrifugation

^{3}H-labelled A26 and ^{14}C-labelled Sp$^-$H12-3 DNA preparations were mixed with a CsCl stock solution to give a final density of 1.695 g/cc, and centrifuged in an SW 50 rotor at 35,000 r.p.m. for 48 hours at 20°C. Following centrifugation the bottom of nitrocellulose tube was punctured and 4-drop fractions were collected. Densities of the fractions were measured according to the method of Schildkraut *et al.* (104). The radioactivity of each fraction was determined by the methods described earlier.

b) Hg-Cs_2SO_4 density gradient centrifugation

The technique described by Skalka *et al.* (108) was used in this study. ^{3}H-labelled A26 DNA (50 μg) was mixed with ^{14}C- labelled Sp$^-$H12-3 (150 μg) in 2.5 ml of 0.1 M Na_2SO_4 solution in a "micro" flask of VirTis homogenizer. The mixture was sheared with the homogenizer at 33,000 r.p.m. for 20 minutes. Under these conditions, the sheared DNA

fragments were of molecular weight about 1.5 x 10^6 daltons (Yamagishi, personal communication). In a nitrocellulose centrifuge tube (7.5 cm x 1.7 cm) the following chemicals were added in the following order: Cs_2SO_4 (2.52 g), DNA mixture (2.5 ml), 0.1 M sodium borate solution (0.125 ml), $HgCl_2$ solution at 100 µg/ml (volume added depended on the rf value used, and would be described below), and 0.1 M Na_2SO_4 - 0.005 M sodium borate solution (amount added to give a final weight of 6.04 g). This mixture contained 41.7% (W/W) Cs_2SO_4. The rf value (defined as mole Hg/mole nucleotide) used was 0.19 and the amount of $HgCl_2$ needed for this preparation was calculated by the following formula: concentration of DNA in µg (in this case 200) x 0.7882 (a constant factor) x rf value (in this case 0.19) = 29.95 µg. The gradient was covered with mineral oil to the point where the tube was almost full and centrifuged in a type 50 titanium rotor at 35,000 r.p.m. for 48 hours at 4°C. Four-drop fractions were collected and the amount of radioactivity in each fraction was determined.

17. Extraction of acid-soluble nucleotides

The method described by Leitzmann and Bernlohr was used to extract TCA-soluble nucleotides (56). The procedure was as follows: nucleotides were extracted from 10 g (wet weight) of cells with 150 ml of cold 5% TCA by manually shaking at 4°C for 15 minutes. The treated cells were

collected by centrifugation and ground with an equal amount of quartz sand. Ground cells were resuspended in 50 ml of cold 5% TCA, shaken for 15 minutes, and centrifuged. The supernatant fluid was combined with the TCA extract obtained earlier, and the pH of the combined solution was adjusted to 5.5 with saturated KOH. To the solution, 5 g of acid-washed Norit A were added, and the mixture was stirred for 2 hours at 4°C to adsorb all the nucleotides. Norit A with bound nucleotides was separated from suspension by centrifugation, washed twice with 50 ml of 0.05 M sodium acetate buffer (pH 4.5), once with distilled water, and then the nucleotides were eluted with cold 50% ethanol containing 1% NH_4OH (50). Five 20 ml elutions were sufficient to remove 90 to 95% of the Norit-bound nucleotides. The eluates were combined, filtered, and the volume was reduced to about 10 ml of vacuum evaporation in a Rotavapor Rotary Vacuum Evaporator at 35°C.

18. Separation and identification of nucleotides

Ion-exchange columns (25 cm x 1.1 cm) were prepared according to the method of Cohn (21) with Dowex-1, formate form. Nucleotide samples (10 ml) were adsorbed on the column at a flow rate of 0.3 ml/minute, and eluted with a formic acid gradient elution system as described by Hurlbert *et al.* (38). Five ml fractions were collected with a fraction collector, and optical density of each

tube was measured at 260 and 275 nm. Ultraviolet (UV)-absorbing materials were pooled and stored in the cold. Most of the samples contained relatively large amounts of formic acid and ammonium formate which interferred with optical density measurement over the UV range. For this reason the formate was removed by evaporating the samples at 35°C in a rotary evaporator. To protect nucleotides from the drop in pH caused by evaporation, 5 to 7 drops of 1 M KOH were added to neutralize formic acid. When the volume has decreased to about 1/5 of the original sample volume, 10 to 15 ml of distilled water were added and the samples again evaporated to a small volume. Samples were then removed with a Pasteur pipette and optical densities were measured at 260 and 275 nm. The ratios of $O.D._{275\ nm}/O.D._{260\ nm}$ assisted in the identification of nucleotides, since it is characteristic for each nucleotide (38). The ratios under these conditions for uridine, adenosine, guanosine and cytidine were 0.6, 0.4, 0.75 and 1 - 2 respectively. The major nucleotide peaks, which were tentatively identified from their 275 nm/260 nm ratios were further purified with the ammonium formate system (38). The rechromatogramed fractions were pooled and formate was removed by evaporation as described above. Ultraviolet absorption spectra of the final products were determined with a Gilford spectrophotometer over the wavelength range of 210 to 300 nm, at pH values of 2, 7, and 11.

19. Paper chromatography of nucleotides

The identity of purified nucleotides was further confirmed by paper chromatography (110). Aliquots of 10 to 20 µl samples were applied on Whatman No. 1 paper, and developed with a mixture of 95% ethanol and 1 M ammonium acetate (7.5:3.0, V:V), pH 7.4. Separation of nucleotides was carried out by a descending method at 20°C for 20 hours.

20. Separation of sugar moiety from UDP derivatives

The sugar moiety was separated from UDP derivatives by the technique of Cabib *et al.* (18). Samples were hydrolyzed with 0.1 N sulphuric acid at 100°C for 10 minutes. The hydrolysates were neutralized with 0.3 N barium hydroxide, and free UDP was precipitated by the addition of an equal volume of 5% zinc sulphate and 0.3 N barium hydroxide. After centrifugation at 7,000 x g for 10 minutes the supernatant fluid contained the sugars.

21. Identification of sugars

a) N-acetylglucosamine

The technique described by Aminoff *et al.* (2) was used. A mixture of 1 ml of the sugar solution and 0.1 ml of 0.5 N Na_2CO_3 solution was heated in a boiling water-bath for exactly 4 minutes, and cooled immediately in an ice-water bath. Glacial acetic acid was added to yield a total volume of 7 ml. One ml of p-dimethylaminobenzaldehyde

(2% solution in glacial acetic acid containing 2.5% of 10 N HCl) and 2 ml of glacial acetic acid were then added and mixed thoroughly. After standing for 1.5 hours at room temperature in the dark, the absorption spectra of the reaction mixtures were determined over the wavelength range of 520 to 620 nm.

b) Galactose and glucose

The cysteine-sulphuric acid technique of Ashwell (5) was applied for the qualitative and quantitative determination of these two sugars. One ml of sugar solution was added into 4.5 ml of sulphuric acid (concentrated sulphuric acid: water, 6:1), and the mixture was shaken in an ice-water bath. Five minutes later the mixture was transferred to room temperature and heated in a boiling water-bath for 3 minutes. After rapid cooling, 0.1 ml of 3% cysteine solution was added, and shaken with a Vortex stirrer for 1 minute. The mixture was let stand at room temperature for 5 minutes before measuring its optical density at 415 and 380 nm. The amount of sugar was determined from the difference in optical densities at 415 and 380 nm. The mixture was further allowed to stand at room temperature for 48 hours at which time the sugars could be identified from their specific colour: blue for galactose and green for glucose.

c) Paper chromatography

This was carried out by the technique of Smith (109). A mixture of isopropanol and water (160:40) was used as solvent. The developing reagent was aniline-diphenylamine reagent (10 volumes of 1% aniline + 1% diphenylamine in acetone and 1 volume of 85% phosphoric acid) which differentiates sugars from the colour developed. Sugars were separated on Whatman No. 4 paper by the descending technique for 20 hours at room temperature. After drying in air, the paper was dipped in the developing reagent and dried again in air. The dried chromatogram was heated in an oven at 100°C for 5 to 7 minutes to obtain specific colour of each sugar.

22. Assay of inorganic phosphorus in nucleotides

Total phosphorus in nucleotides was liberated by hydrolysis in 1 N sulphuric acid at 100°C for 30 minutes and acid-labile phosphorus was liberated by hydrolysis in 0.1 N sulphuric acid at 100°C for 20 minutes (20). The hydrolyzed sample (0.3 ml) was introduced to a small test tube containing 0.7 ml of the reagent (10% ascorbic acid: 0.42% ammonium molybdate.$4H_2O$ in 1 N sulphuric acid, 1:6) and incubated at 45°C for 20 minutes. The intensity of colour developed was measured at 820 nm.

23. Analysis of cellular nucleic acids

Cells were grown in SSM and collected by

centrifugation at 7,000 x g for 10 minutes. After washing in 1 x SSC, DNA and RNA were extracted and separated from each other by the method of Schmidt and Thannhauser modified by Siminovitch and Graham (106). One g of cells (wet weight) was suspended in 30 ml of 5% TCA and kept in an ice-water bath for 1 hour. After centrifugation, the sediment was washed once with 30 ml of 5% TCA, once with 30 ml of cold absolute ethanol, and twice with 30 ml of boiling absolute ethanol for periods of 15 minutes. Six ml of 0.3 N KOH were added to the residue and the mixture was allowed to stand for 18 hours at 37°C to complete hydrolysis. The hydrolysate was made slightly acidic with 6 N HCl and then 5% with respect to TCA. After centrifugation, the amount of RNA in the supernatant fluid was determined by Mejbaum's orcinol reaction (69). The residue, containing polymerized DNA, was resuspended in 2 to 3 ml of 0.1 N HCl, precipitated with 5% TCA, extracted once with 3 ml of 5% TCA at 90°C for 15 minutes and centrifuged. The supernatant solution thus obtained was used to measure DNA concentration by the diphenylamine reaction (17).

24. Extraction of enzymes

The method of Gunsalus (31) was used to prepare crude extracts. Cells (1 g, wet weight) were ground with quartz sand and suspended in 5 ml of 0.15 M KCl. After centrifugation at 7,000 x g for 10 minutes, the supernatant

fluid was kept frozen at -40°C. Extracts were partially purified by adsorption with bentonite and by ammonium sulphate precipitation (125,131). To 1 ml of crude extract, 50 mg of bentonite were added, stirred for 5 minutes, and centrifuged at 7,000 x g for 10 minutes. The supernatant fluid obtained was mixed with an equal volume of saturated ammonium sulphate solution, centrifuged again and to the supernatant fluid 0.25 volume of saturated ammonium sulphate solution was added. The precipitate formed was dissolved in 1/20 of the original volume of solution in 0.15 M KCl. This was used as the partially purified enzyme preparation and stored at -40°C.

25. Determination of protein content in the enzyme preparations

The technique of Lowry *et al.* (60) was used for the determination of protein content in the enzyme preparations. To 0.2 ml of enzyme preparations, 1.0 ml of a mixture containing 2% Na_2CO_3 solution in 0.1 N NaOH and 0.5% $CuSO_4.5H_2O$ solution in 1% sodium tartrate (50:1) was added, and let stand at room temperature for 15 minutes. Then 0.1 ml of diluted (1 N) Folin-Ciocalteau phenol reagent was added to the above solution. The mixture was shaken immediately with a Vortex stirrer, let stand for 30 minutes at room temperature and the optical density was read at 600 nm. A standard curve was constructed with bovine serum albumin solutions.

26. Assay of UDP - galactose-4-epimerase

Partially purified enzyme preparations were used to assay the epimerase activity by the technique of Maxwell *et al.* (67). The assay method was as follows: 50 µl of glycine buffer (1 M, pH 8.8), 10 µl of UDP - galactose (5 mM), 10 µl of DPN (50 mM), 20 µl of UDPG dehydrogenase and 0.5 ml of distilled water were mixed in a quartz cuvette (light path = 1 cm). The optical density was measured at 340 nm immediately after mixing. Five minutes later the optical density was measured again at the same wavelength in order to check the possible contamination of UDPG in the reagents used. Then 0.02 ml of the enzyme preparation was added and mixed immediately. The rate of increase in optical density at 340 nm was followed for 4 minutes at 1 minute intervals. The rate of reaction was proportional to the enzyme concentration up to an increase in optical density of about 0.050 per minute (67). A unit of enzyme activity will produce an increase in optical density at 340 nm of 0.001 per minute under the above conditions.

27. Assay for galactose-1-phosphate uridyl transferase

The method of Maxwell *et al.* (67) was employed for assaying the transferase activity in partially purified enzyme preparations. The assay procedure was as follows: 60 µl of glycine buffer (0.1 M, pH 8.8), 30 µl of cysteine

buffer (0.16 M, pH 8.5), 5 µl of $MgCl_2$ (1 M), 20 µl of TPN (0.02 M), 25 µl of UDPG (0.01 M), 10 µl of glucose-1, 6-diphosphate (0.175 M), 10 µl of phosphoglucomutase (70 units/ml), 5 µl of glucose-6-phosphate dehydrogenase (14 units/ml), 425 µl of distilled water and 10 µl of the partially purified enzyme preparation was added to a quartz cuvette (light path = 1 cm). Readings were taken at 340 nm at 1 minute intervals after addition of 20 µl of galactose-1-phosphate (0.0175 M). A unit of enzyme activity will produce an increase in optical density of 340 nm of 0.001 per minute under the above conditions.

CHAPTER III

DELETION IN STRAIN $SP^{-}H12-3$

The objective of this work was to characterize by physical techniques the deletion in the chromosome of asporogenous strain $Sp^{-}H12-3$.

A. Messenger RNA for Sporulation

Investigations on m-RNA for sporulation initiated by Yamagishi and Takahashi (136) were further extended by the use of improved techniques.

1. Unstable RNA

Populations of RNA in bacterial cells are composed of m-RNA, ribosomal-RNA and transfer-RNA molecules. While the last two RNA species are considered to be stable most m-RNA species usually undergo rapid degradation and resynthesis, i.e., turnover. Since actinomycin D inhibits DNA-directed RNA synthesis (91), the amount of unstable RNA may be estimated by measuring the amount of trichloroacetic acid (TCA) precipitable RNA after the addition of this antibiotic. In this study, the changes of m-RNA in strains A26 (sp^{+}), $Sp^{-}H12-3$ (a stage 0, sp^{-} mutant) and $Sp^{-}N2-2$ (a stage II, sp^{-} mutant) have been

determined at various stages of growth.

^{3}H-uridine was added to cultures of A26, Sp^{-}H12-3 or Sp^{-}N2-2 at log, t_0, t_1, t_2 or t_3 stage. Six minutes after the addition of ^{3}H-uridine, one half of each culture was removed to another culture flask to which actinomycin D was added. Radioactivities incorporated into the actinomycin D treated or untreated cells were measured at intervals. Results are shown in Figs. 1 to 5. All strains tested showed practically the same rate of ^{3}H-uridine incorporation and the same amount of ^{3}H-RNA chased by actinomycin D during log-phase and at t_0. At t_1, however, the amount of ^{3}H-uridine incorporated and ^{3}H-RNA chased in Sp^{-}H12-3 became much smaller than that in A26 and Sp^{-}N2-2. At t_2 the amount of unstable RNA in Sp^{-}N2-2 was still comparable to that of sporulating culture (A26) and only at t_3 Sp^{-}N2-2 showed a reduced amount of ^{3}H-RNA chased by actinomycin D.

The results are in agreement with the cytological observation that Sp^{-}H12-3 is a stage 0 mutant, and Sp^{-}N2-2 a stage II mutant.

2. DNA-RNA hybridization

Since Sp^{-}H12-3 appears to have a block at a very early stage of sporulation, Takahashi (124) has proposed a working hypothesis that this is a mutant of regulation and that the m-RNA, which is absent in this strain at t_1, may

Fig. 1. Incorporation and chasing of ^{3}H-uridine in *B. subtilis* strains A26, Sp^{-}H12-3 and Sp^{-}N2-2 in the log-phase.

Cells were grown in Schaeffer's sporulation medium and ^{3}H-uridine at a final concentration of 5 μc/ml was added to the cultures in the log-phase. Six minutes after the addition of ^{3}H-uridine, one-half of the cultures were transferred into into another culture flask to which actinomycin D was added at a final concentration of 10 μg/ml. Samples (0.2 ml) were taken into tubes containing 10 ml of 5% TCA, kept in an ice-water bath for 1 hour, and filtered through a nitrocellulose membrane filter (S & S, No. B-6). Membrane filters were dried under an infrared lamp, put into a vial containing 10 ml of scintillation fluid and counted in a Beckman Model LS-250 liquid scintillation counter.

a - A26

b - Sp^{-}H12-3

c - Sp^{-}N2-2

O: no actinomycin D

●: with actinomycin D

Each point represents the average value of three determinations.

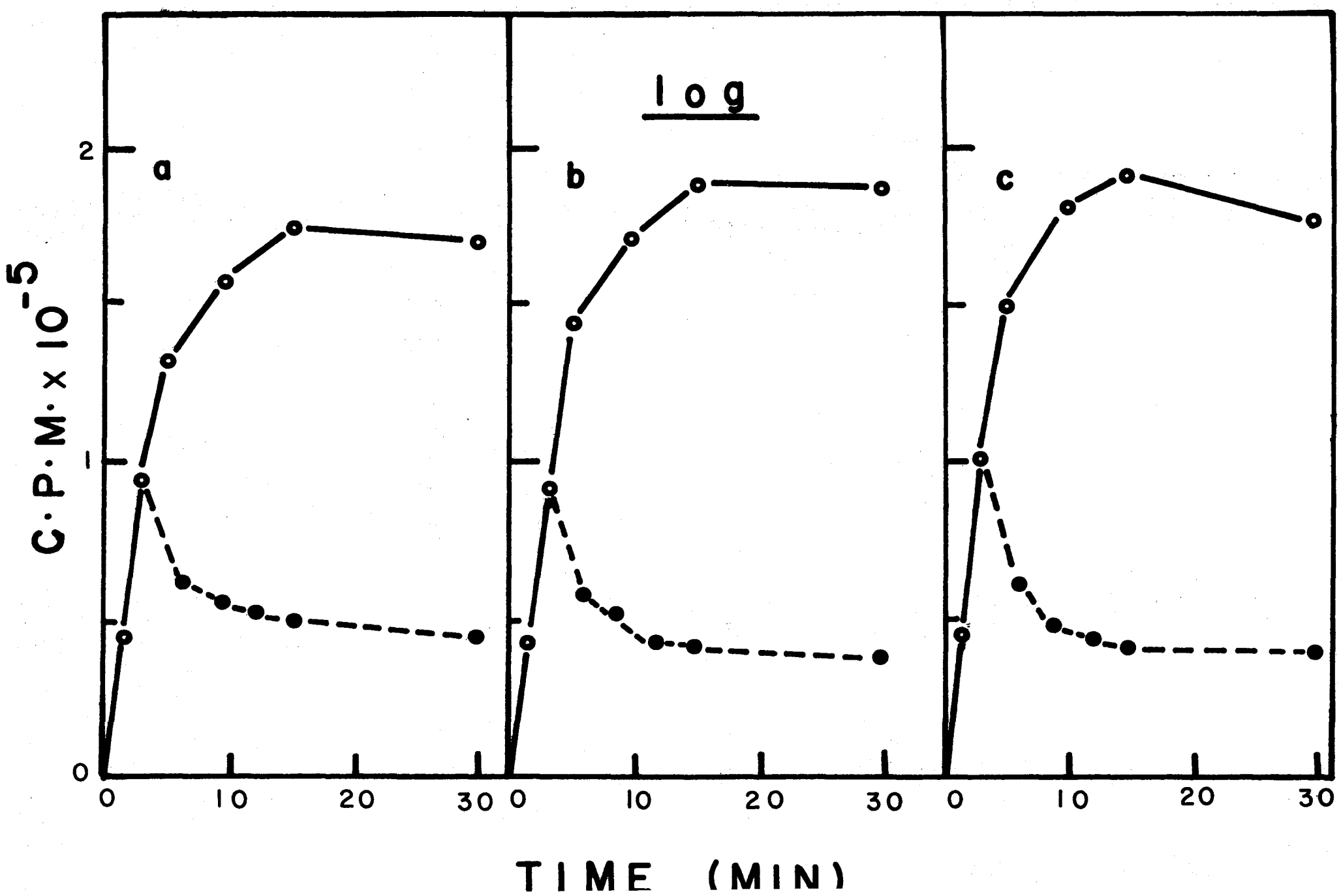

log
a
b
c
C·P·M·x 10⁻⁵
2
1
0
0
10
20
30
TIME (MIN)

Fig. 2. Incorporation and chasing of ^{3}H-uridine in *B. subtilis* strains A26, Sp^{-}H12-3 and Sp^{-}N2-2 at t_0.

The legend is the same as in Fig. 1, except that ^{3}H-uridine was added at t_0 instead of in the log-phase.

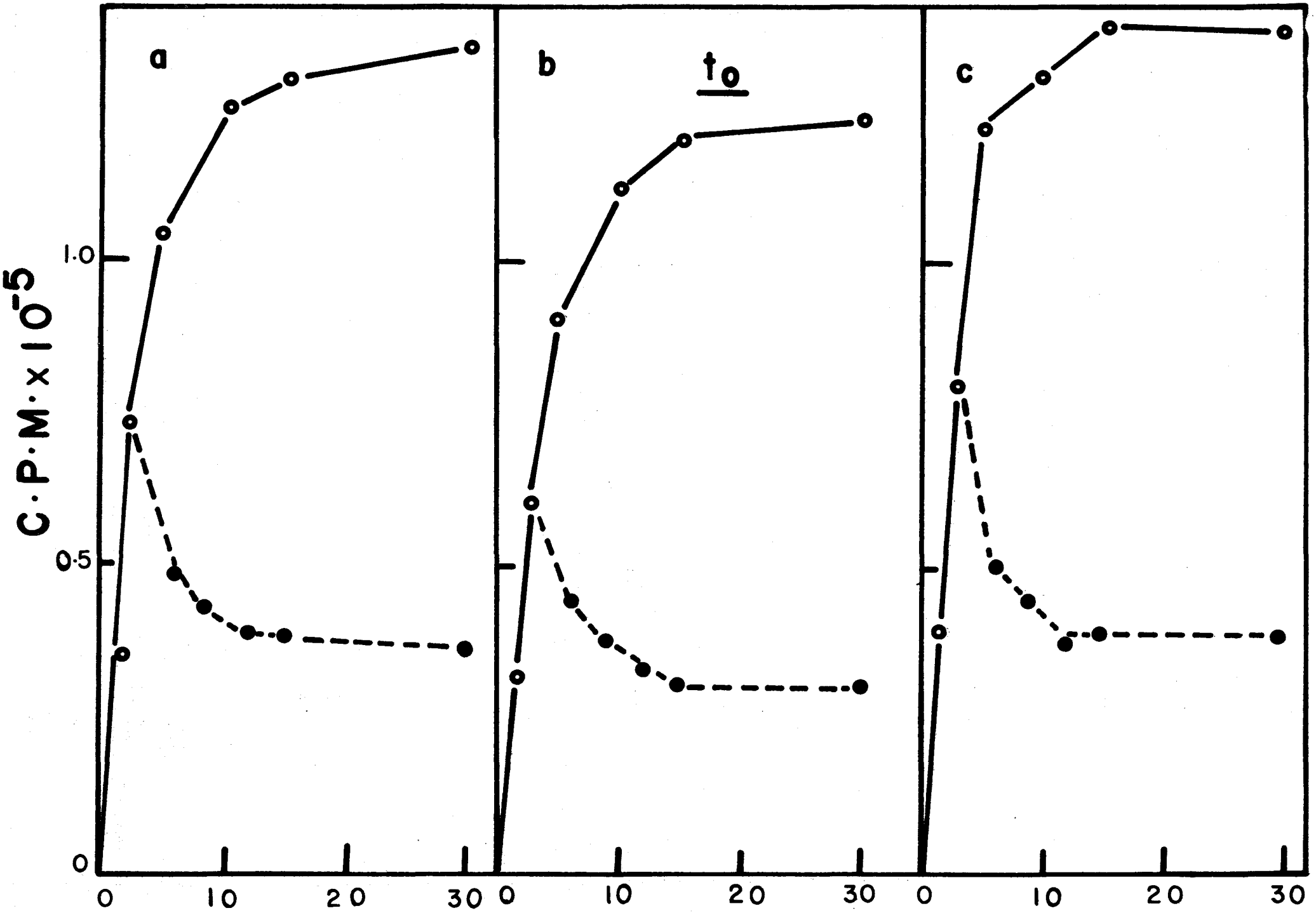

a
b
t_o
c
C·P·M·x 10^{-5}
1.0
0.5
0
0
10
20
30
0
10
20
30
0
10
20
30

Fig. 3. Incorporation and chasing of ^{3}H-uridine in *B. subtilis* strains A26, Sp^{-}H12-3 and Sp^{-}N2-2 at t_1.

The legend is the same as in Fig. 1, except that ^{3}H-uridine was added at t_1 instead of in the log-phase.

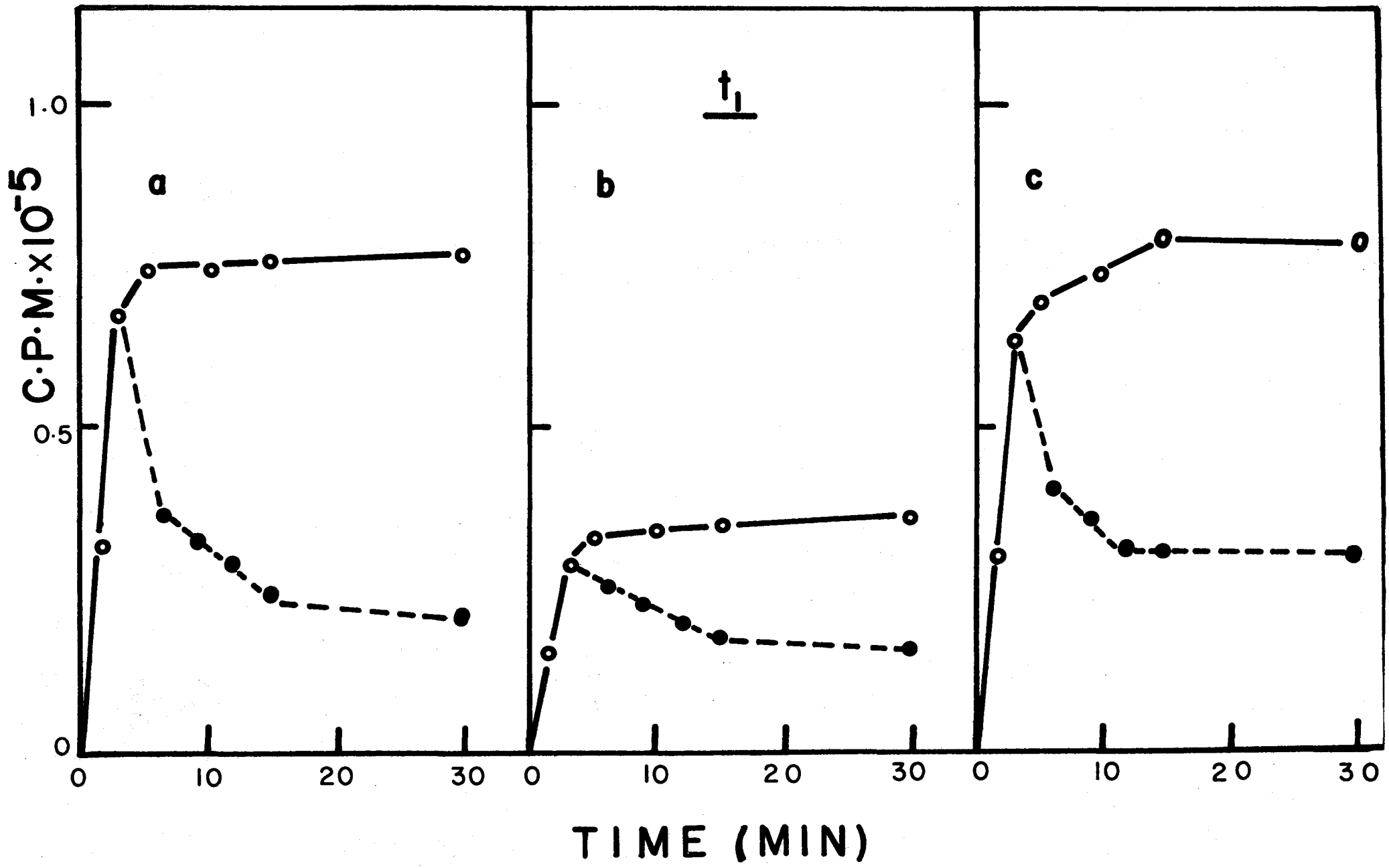

1·0
0·5
0
C·P·M·×10⁻⁵
a
b
c
t1
0
10
20
30
TIME (MIN)

Fig. 4. Incorporation and chasing of ^{3}H-uridine in *B. subtilis* strains A26, Sp^{-}H12-3 and Sp^{-}N2-2 at t_2.

The legend is the same as in Fig. 1, except that ^{3}H-uridine was added at t_2 instead of in the log-phase.

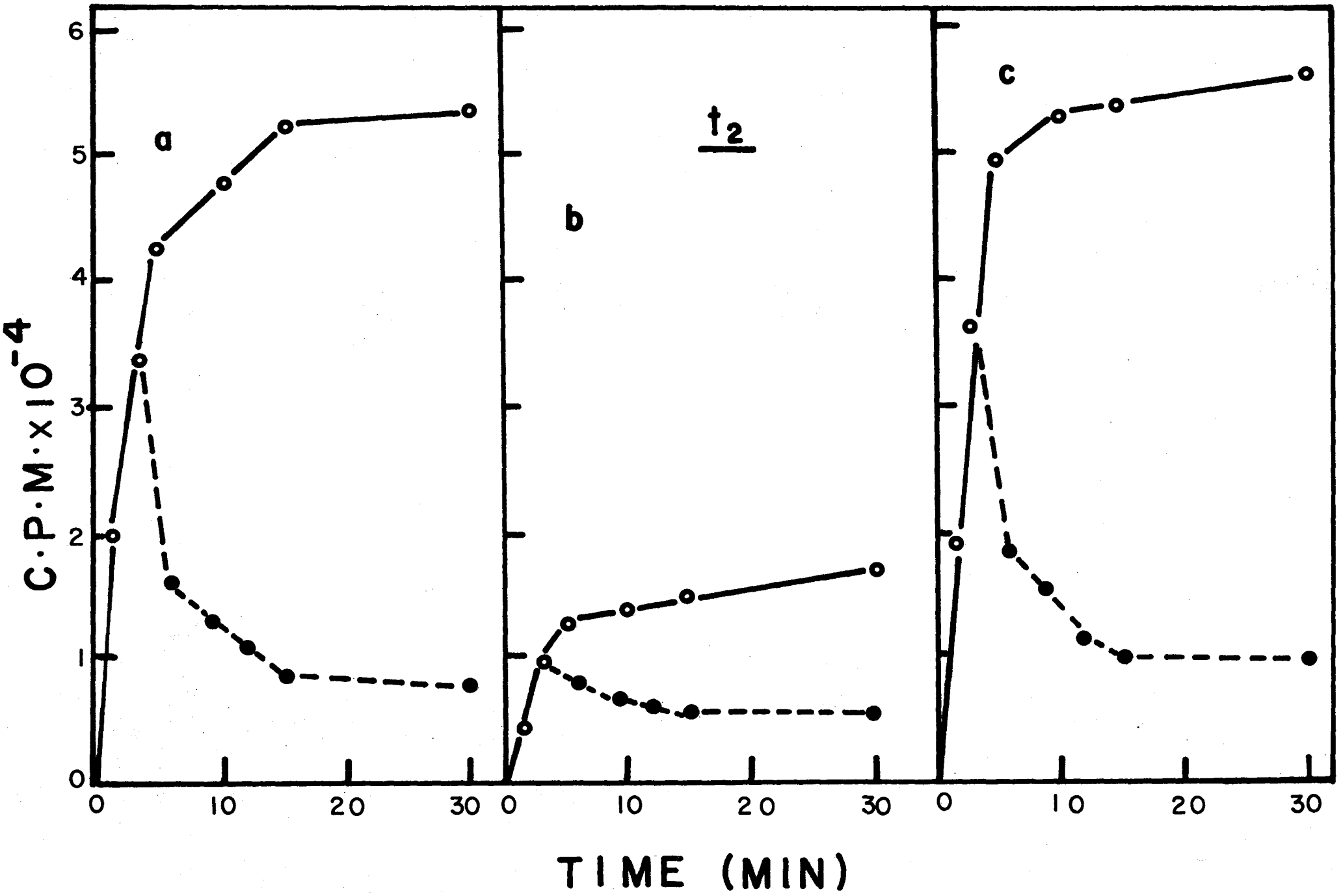
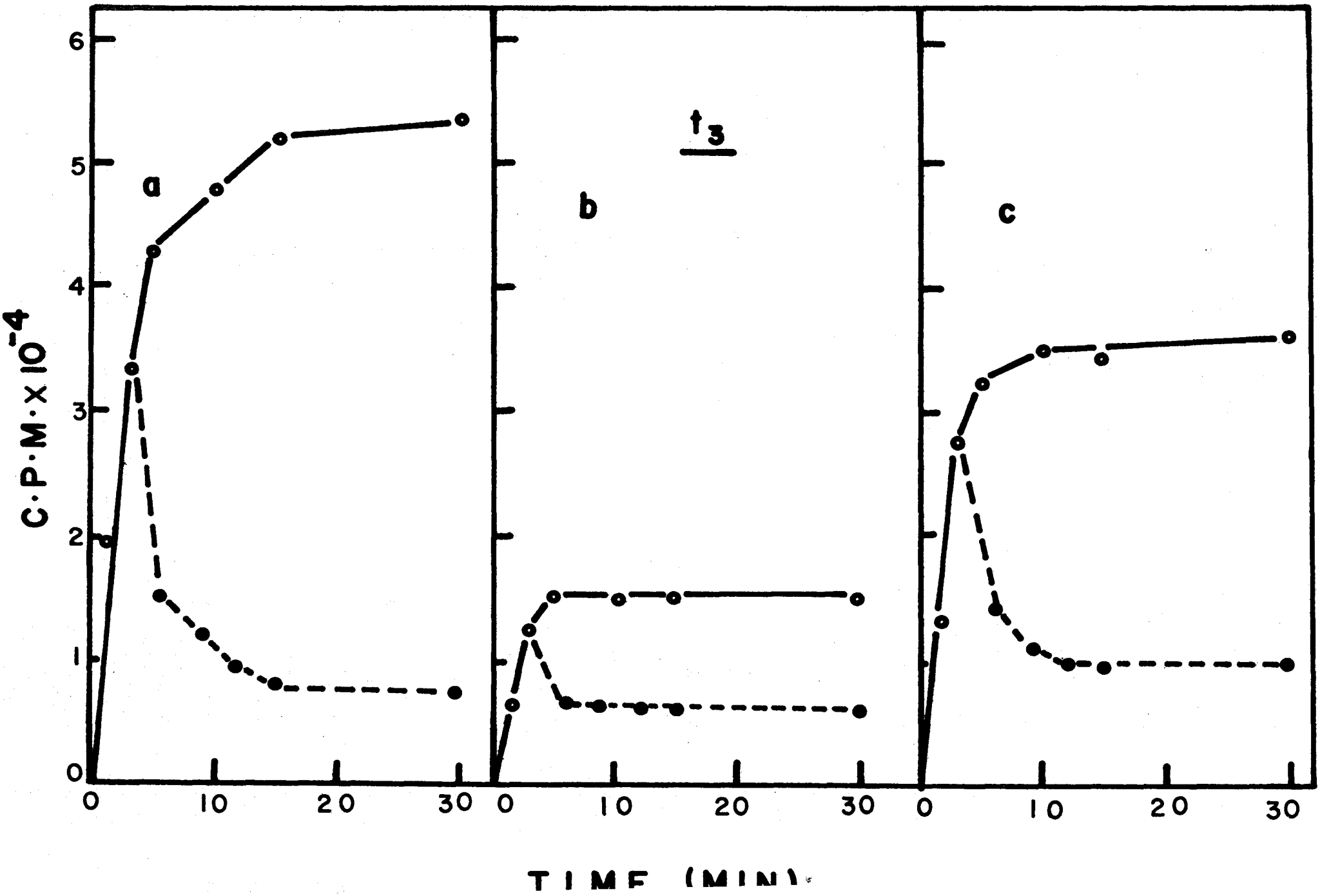

a
b
c
t_2
C·P·M·×10^{-4}
0
1
2
3
4
5
6
0
10
20
30
TIME (MIN)

Fig. 5. Incorporation and chasing of ^{3}H-uridine in *B. subtilis* strains A26, Sp^{-}H12-3 and Sp^{-}N2-2 at t_3.

The legend is the same as in Fig. 1, except that ^{3}H-uridine was added at t_3 instead of in the log-phase.


a
b
c
t_3
C·P·M·X 10⁻⁴
0
1
2
3
4
5
6
0
10
20
30
TIME (MIN)

be important in the initiation of sporulation. In order to study this population of m-RNA in detail, an improved hybridization technique has been used in the present study. The hybridization technique originally used by Yamagishi and Takahashi is a modified method of Nygaard and Hall (81, 136) in which both RNA and denatured DNA are in solution. In this procedure, some of the single-stranded DNA molecules inevitably reanneal during hybridization. As a consequence, the amount of DNA hybridizable with RNA in the annealing mixture becomes smaller and at the same time the hybridization efficiency (RNA hybridized/RNA input) becomes lower. This problem has been eliminated by Gillespie and Spiegelman (28). Their method involves immobilization of denatured DNA molecules on nitrocellulose membranes, and hybridization takes place on the membrane between the immobilized DNA and RNA in solution. As suggested by Yamagishi and Takahashi (136) the presence of a relatively large amount of m-RNA for vegetative functions at a relatively early stage of sporulation may mask hybridization of sporulation m-RNA. Attempts were therefore made to separate sporulation m-RNA from other species of RNA by centrifugation in a sucrose gradient. The sedimentation profile of ^{3}H-A26 t_2 RNA in a sucrose gradient is shown in Fig. 6. The fractionated RNA samples were then analyzed by the membrane technique of Gillespie and Spiegelman (28). As illustrated in Fig. 7, RNA in fractions 17, 18, 19 and

Fig. 6. Sedimentation profile of ^{3}H-labelled A26 t_2 RNA in a sucrose gradient.

Two-tenth ml of ^{3}H-A26 t_2RNA was layered on the top of a linear sucrose gradient (4.7 ml, 15-30%) and centrifuged at 35,000 r.p.m. for 15 hours in an SW 50 rotor at 4°C in a Beckman L2-65 ultracentrifuge. Four-drop fractions were collected from the bottom of the centrifuge tube in tubes containing 1 ml of 2 x SSC. Samples (0.05 ml) were taken for radioactivity determination and the rest was used for optical density measurement at 260 nm.

O: O.D. at 260 nm

●: radioactivity/0.05 ml of samples.

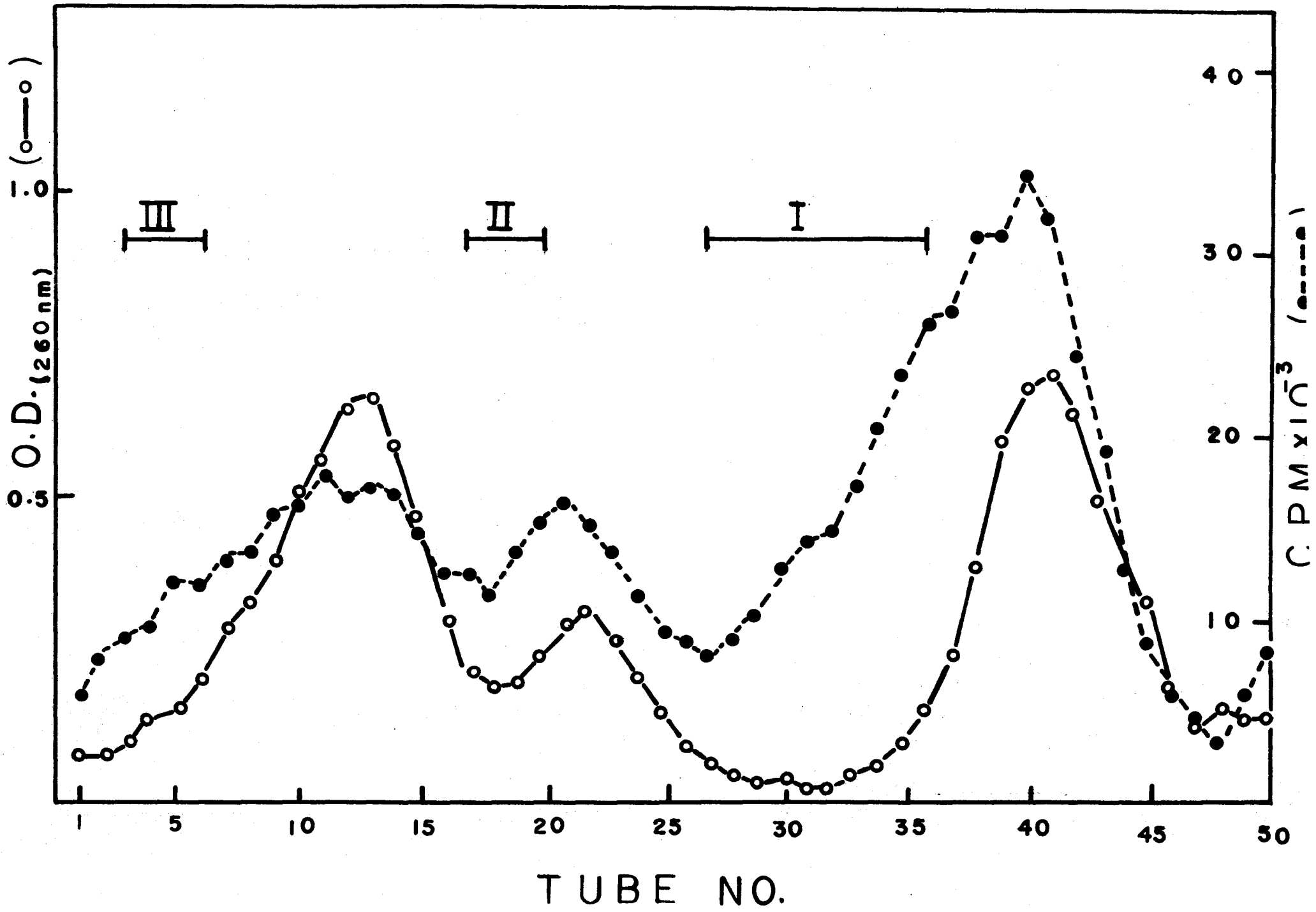

O.D.(260 nm) (o—o)
CPM x 10^-3 (•---•)
III
II
I
1.0
0.5
40
30
20
10
1
5
10
15
20
25
30
35
40
45
50
TUBE NO.

Fig. 7. Enrichment of m-RNA by absorption on $Sp^{-}H12-3$ DNA.

^{3}H-A26 t_2 RNA was fractionated in a linear sucrose gradient (see Fig. 6). One µg of RNA from each fraction was hybridized with 100 µg of DNA fixed on a nitrocellulose membrane.

C.P.M.(A): the amount of radioactive hybrid formed with A26 DNA.

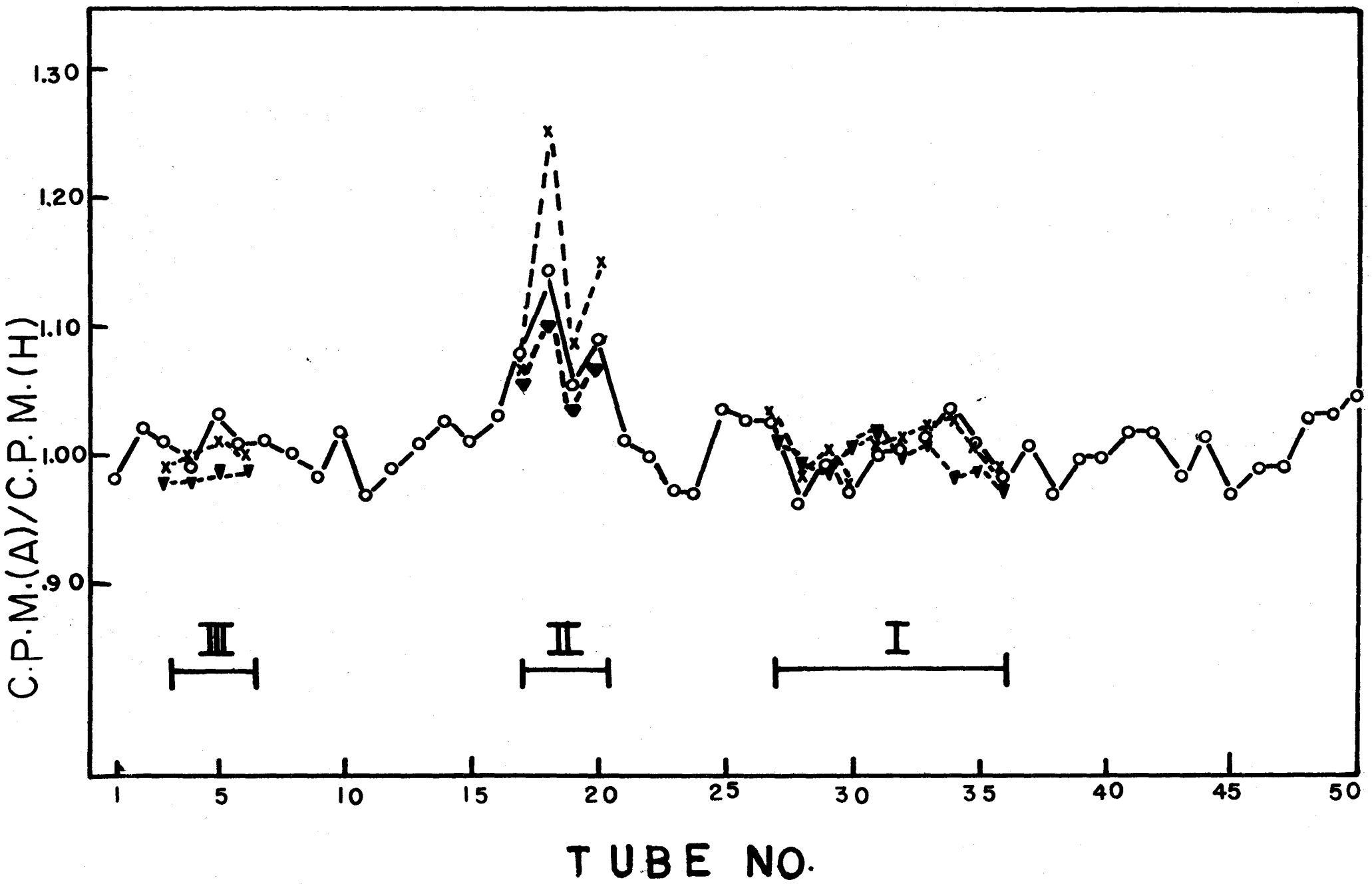
C.P.M.(H): the amount of radioactive hybrid formed with $Sp^{-}H12-3$ DNA.

O: "untreated" A26 t_2 RNA (RNA not absorbed by any DNA).

X: "$Sp^{-}H12-3$ treated" A26 t_2 RNA (RNA absorbed by $Sp^{-}H12-3$ DNA).

▼: "A26 treated" A26 t_2 RNA (RNA absorbed by A26 DNA)

The specific radioactivities of the "untreated" and "$Sp^{-}H12-3$" or "A26" treated RNA were in the range of 10,000 - 160,000 c.p.m./µg and 3,000 - 4,500 c.p.m./µg, respectively. About 55% (for "untreated" RNA) or 15% (for "treated" RNA) of the input radioactivity were hybridized under these conditions.
Each point represents the average value of four determinations.


1.30
1.20
1.10
1.00
.90
C.P.M.(A)/C.P.M.(H)
III
II
I
1
5
10
15
20
25
30
35
40
45
50
TUBE NO.

20 formed 5 to 10% more radioactive hybrids with A26 DNA than with Sp^-H12-3 DNA. These fractions of RNA sedimented between 16S and 23S RNAs.

This marked difference in the amount of hybrid formed was not observed with ^3H-A26 RNA extracted at t_0 or t_1. According to Yamagishi and Takahashi (136), a species of m-RNA for sporulation which is detectable of hybrid competition experiments is synthesized at t_1. In the present study, to obtain a better incorporation of ^3H-uridine, uracil was omitted from the sporulation medium used by Yamagishi and Takahashi (136). It is therefore probable that the stage t_1 in their experiment corresponds to the stage t_2 in the present experiment.

Sporulation m-RNA was further enriched by the following technique. One µg of ^3H-A26 t_2 RNA from the fractions which showed relatively high specific radioactivities Group (Gr.) I, II and III in Fig. 6, was absorbed by Sp^-H12-3 DNA fixed on a membrane (100 µg/membrane). This absorption procedure was repeated once more after an incubation of 16 hours. This technique was based on the idea that certain species of m-RNA would not hybridize with Sp^-H12-3 DNA and remain unabsorbed because of deletion in the DNA. The portion of RNA which was not absorbed by Sp^-H12-3 DNA was designated as "Sp^-H12-3 treated ^3H-A26 t_2 RNA", and the RNA preparation which was not absorbed by this procedure was designated as "untreated RNA". When

"Sp$^-$H12-3 treated ^{3}H-A26 t_2 RNA" was hybridized with A26 or Sp$^-$H12-3 DNA, the amount of RNA (Gr. II) hybridized with A26 DNA was 10 to 20% higher than that hybridized with Sp$^-$H12-3 DNA. For other fractions of "Sp$^-$H12-3 treated A26 t_2 RNA" (in the regions of Gr. I and III), almost the same amount of RNA hybridized with A26 and with Sp$^-$H12-3 DNA (Fig. 7). A control experiment was performed with "A26 treated ^{3}H-A26 t_2 RNA" which was prepared by absorbing ^{3}H-A26 t_2 RNA with A26 DNA by the procedure described earlier. In this case, as expected the amount of RNA hybridized with A26 and that hybridized with Sp$^-$H12-3 was very similar (Fig. 7).

3. DNA-RNA hybrid competition

Messenger RNA for sporulation was further analyzed by hybrid competition experiments in which ^{3}H-A26 t_2 RNA was hybridized with A26 DNA in the presence of increasing amounts of unlabelled RNA. The amount of ^{3}H-A26 t_2 RNA hybridized with A26 DNA in the absence of unlabelled RNA was considered as 100% hybridized radioactivity, and the reduction in percent hybridized radioactivity in the presence of unlabelled RNA was determined (Fig. 8). It was observed that the amount of radioactive hybrids formed between A26 DNA and A26 t_2 Gr. I or III RNA was reduced to the same extent by the addition of unlabelled t_2 RNA extracted from A26 or Sp$^-$H12-3 (Figs. 8a and 8c). In contrast, when A26 t_2 Gr. II RNA was used, unlabelled RNA extracted at t_2 from Sp$^-$H12-3 competed about 11% less than

Fig. 8. Competition between ^{3}H-A26 t_2 RNA and unlabelled A26 or Sp^{-}H12-3 t_2 RNA on DNA sites.

Hybridization mixtures contained a nitrocellulose membrane on which 10 µg of A26 DNA were fixed, indicated amount of unlabelled A26 t_2 (●) or Sp^{-}H12-3 t_2 (O) RNA, and

a - 0.25 µg of A26 t_2 Gr. I RNA (109,000 c.p.m./µg),

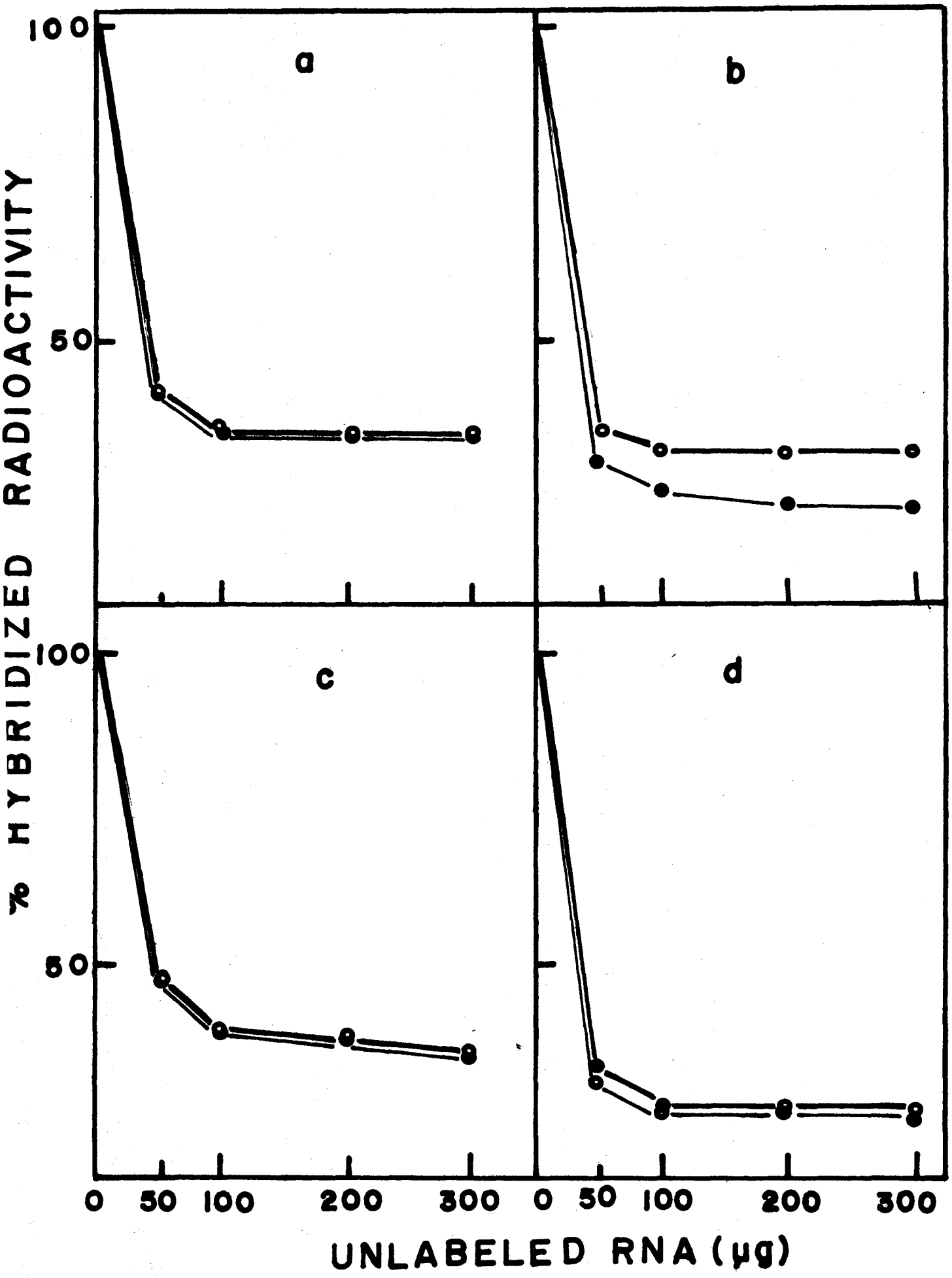
b - 0.45 µg of A26 t_2 Gr. II RNA (30,000 c.p.m./µg),

c - 0.15 µg of A26 t_2 Gr. III RNA (40,100 c.p.m./µg),

d - same as in (b), except that 10 µg of Sp^{-}H12-3 DNA was used in place of A26 DNA.

In control mixtures (where unlabelled RNA was not added), 51% (a), 49% (b), 46% (c) and 45% (d) of the added ^{3}H-A26 RNA were hybridized with DNA.

Each point represents the average value of six determinations.


a
b
c
d
100
50
100
50
% HYBRIDIZED RADIOACTIVITY
0 50 100 200 300
0 50 100 200 300
UNLABELED RNA (µg)

did RNA extracted at the same stage from A26 cells (Fig. 8b). The difference in competition disappeared when similar experiments were carried out by hybridizing A26 t_2 Gr. II RNA with Sp^-H12-3 DNA (Fig. 8d). Unlabelled RNA extracted from SB19E or Sp^-N2-2 at t_2 showed competing capacity very similar to that of unlabelled A26 t_2RNA. These results are in accordance with the findings of Yamagishi and Takahashi (136).

Hybrid competition experiments were also carried out with "Sp^-H12-3 treated ^{3}H-A26 t_2 Gr. II RNA". The difference in competition between the unlabelled t_2 RNA from A26 and Sp^-H12-3 increased from 11% (as shown in Fig. 8b) to 23% (Fig. 9a). A control experiment was carried out with "A26 treated ^{3}H-A26 t_2 Gr. II RNA". In this case results were similar to those obtained with untreated ^{3}H-A26 t_2 Gr. II RNA (Fig. 9b).

B. The DNA of Mutant Sp^-H12-3

Hershey *et al.* (35) have shown that native DNA molecules can be broken into fragments of relatively uniform sizes by subjecting them to appropriate rates of hydrodynamic shear. Furthermore, Nandi *et al.* (75,127) have introduced the technique of separating DNA fragments of different base composition by Hg-Cs_2SO_4 density gradient centrifugation. This technique is based on the finding that mercuric ions combine preferentially with regions

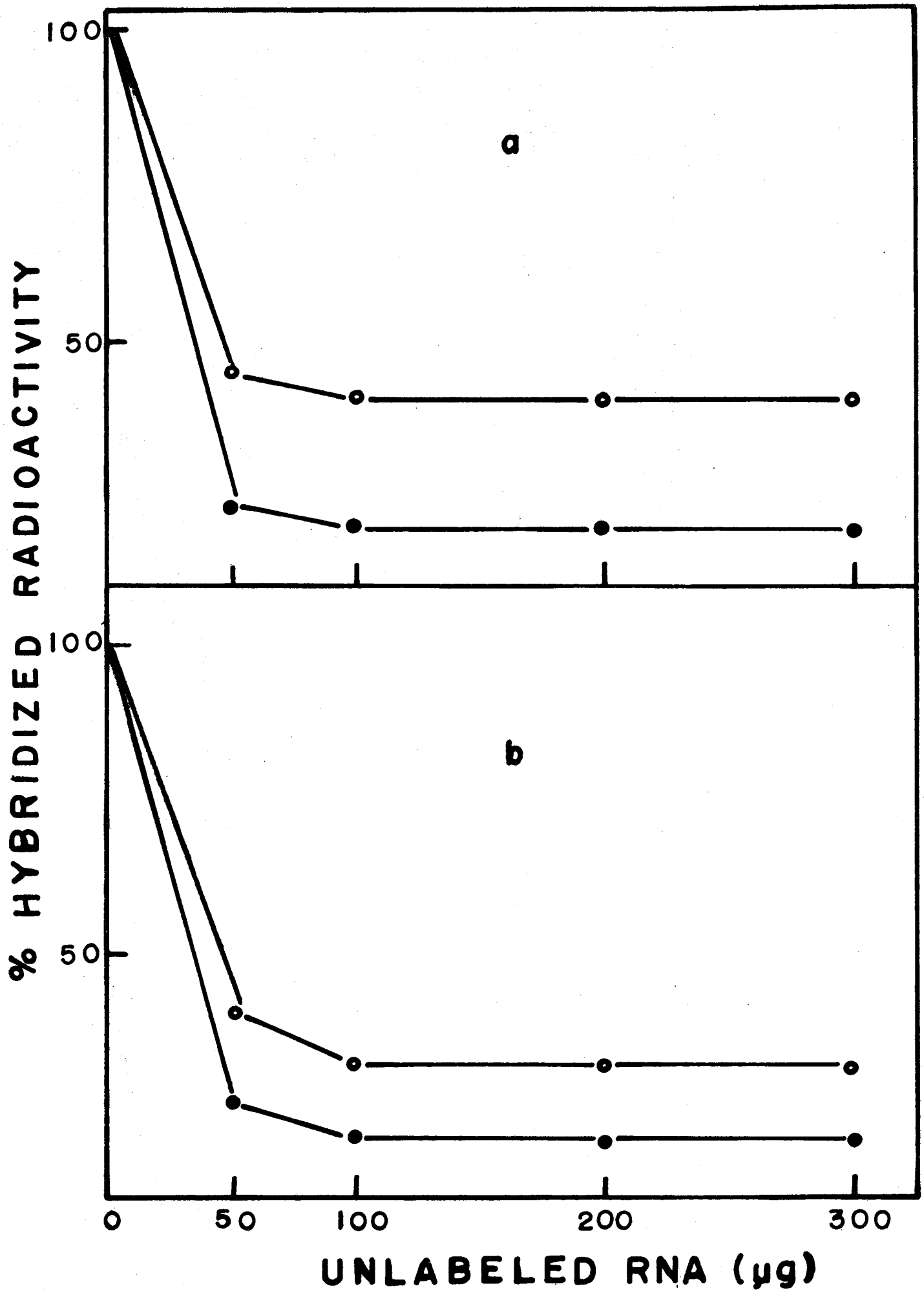
Fig. 9. Competition between the "Sp⁻H12-3 treated" or "A26 treated" ^{3}H-A26 t_2 Gr. II RNA and unlabelled A26 or Sp⁻H12-3 t_2 RNA on A26 DNA sites.

Hybridization mixtures contained a nitrocellulose membrane on which 10 µg of A26 DNA were fixed, indicated amount of unlabelled A26 t_2 (●) or Sp⁻H12-3 t_2 (O) RNA, and

a - 0.45 µg of "Sp⁻H12-3 treated A26 t_2 Gr. II RNA" (11,000 c.p.m./µg),

b - 0.45 µg of "A26 treated A26 t_2 Gr. II RNA" (8,000 c.p.m./µg).

In control mixtures (where unlabelled RNA was not added), 15% (a) and 11% (b) of the added ^{3}H-A26 RNA were hybridized with A26 DNA. Each point represents the average value of six determinations.


100
50
a
% HYBRIDIZED RADIOACTIVITY
100
50
b
0
50
100
200
300
UNLABELED RNA (µg)

rich in adenine and thymine in DNA to form complexes which show an increased buoyant density. By these techniques, Skalka *et al.* (108) have shown that DNA molecules of phage lambda of about 1.5×10^6 daltons prepared by shearing can be separated into fractions with very small difference in guanine and cytosine (G + C) content. Segments of different compositions, ranging from 37 to 57% (G + C) have been found in lambda DNA (108). These segments are reasonably homogeneous internally and the boundaries between segments are sharp. It has been found later that DNAs isolated from *E. coli* (135) and *B. subtilis* (Yamagishi and Takahashi, in press) also contain, in varying degree, segments of dissimilar nucleotide composition. On the other hand, DNAs from the classicially "virulent" types of phages (T2, T4) tend to be internally homogeneous (107).

Nucleotide analysis of the fractionated wild-type *B. subtilis* DNA carried out by Yamagishi and Takahashi (in press) has shown that the G + C content of fragments of 1.5×10^6 daltons varies from 35 to 50%, with a mean and standard deviation of 44 ± 3.4%. Thus it may be possible to demonstrate deletion in Sp^-H12-3 DNA directly by comparing the distribution of DNA fragments from the mutant with those of wild-type strains, if the deletion is of a considerable size. ^{14}C-labelled Sp^-H12-3 DNA and ^{3}H-labelled wild-type DNA were sheared together and banded in a Hg-Cs_2SO_4 gradient. The presence of fractions which show a

$^{3}H/^{14}C$ ratio considerably higher than the average value would be indicative of deletion in $Sp^{-}H12-3$ chromosome.

1. CsCl density gradient centrifugation

The fractionation of DNA fragments in a Hg-Cs_2SO_4 gradient is based on the fact that Hg-ion binds preferentially with A-T pairs in native DNA. With denatured DNA, however, the binding of Hg-ion is less specific (75,127). Therefore, prior to centrifugation in a Hg-Cs_2SO_4 density gradient, the presence of denatured DNA in sheared preparations was checked by centrifuging DNA fragments in a CsCl density gradient. ^{3}H-A26 DNA and ^{14}C-Sp^{-}H12-3 DNA were mixed and sheared in a VirTis homogenizer. An aliquot of the sheared DNA mixture was centrifuged in a CsCl solution. Both ^{3}H-A26 and ^{14}C-Sp^{-}H12-3 DNA banded at a buoyant density of 1.702 g/cc in the gradient (Fig. 10). No significant amount of radioactivity was found at a position corresponding to the density of denatured DNA. The result also suggests that the average G + C base composition of the two DNA species are very similar (95,116). This result also indicates that density increment due to the presence of ^{14}C isotope in our DNA preparation is negligible.

2. Hg-Cs_2SO_4 density gradient centrifugation

A mixture of sheared ^{3}H-A26 DNA and ^{14}C-Sp^{-}H12-3 DNA was centrifuged in a Hg-Cs_2SO_4 solution with an rf value

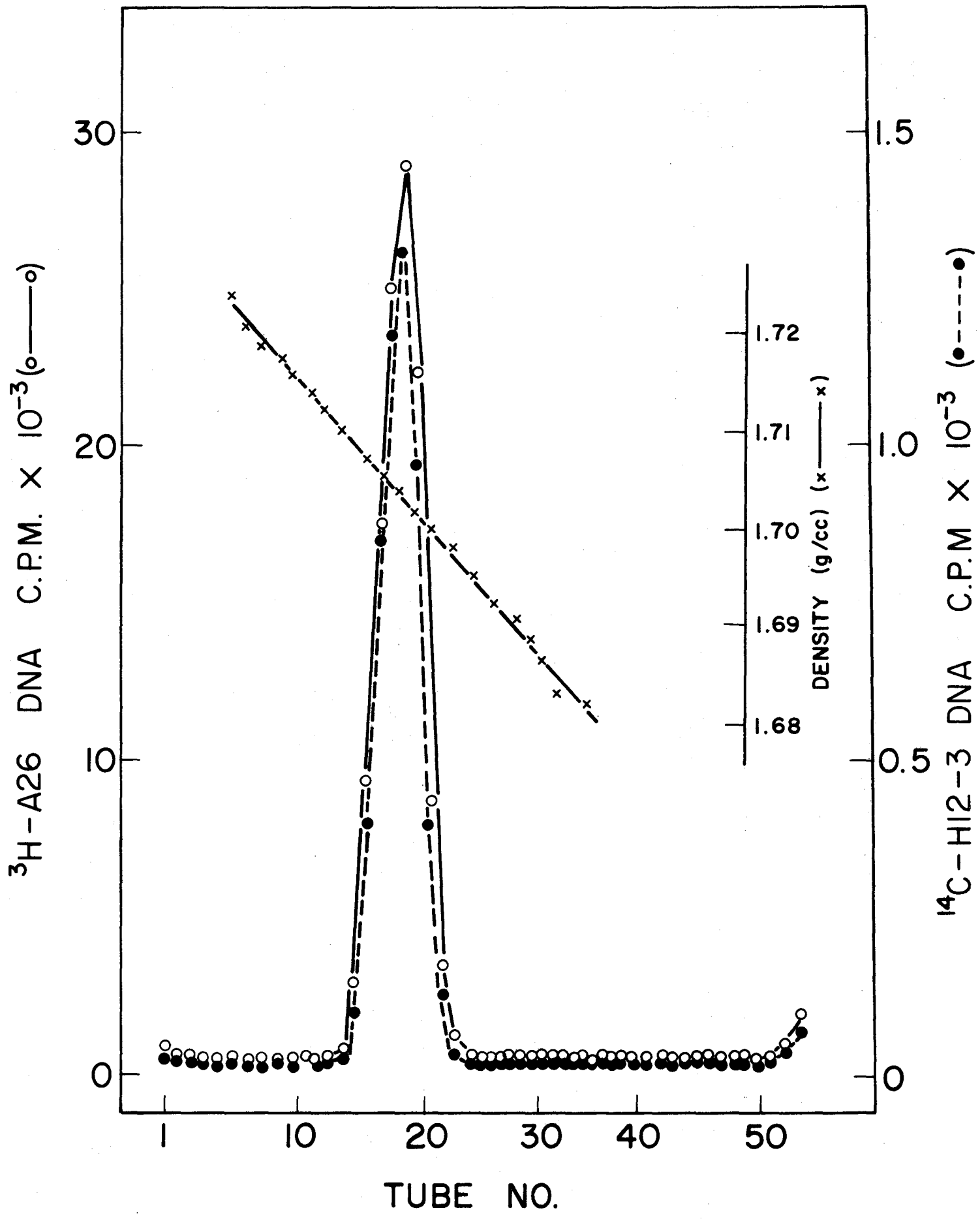
Fig. 10. Distribution of ^{3}H-A26 and ^{14}C-Sp^{-}H12-3 DNA in a CsCl density gradient

^{3}H-A26 DNA (4 µg, 5.5 x 10^{4} c.p.m./µg) and ^{14}C-Sp^{-}H12-3 DNA (10 µg, 752 c.p.m./µg) were mixed, sheared and centrifuged in a CsCl solution (final density = 1.695 g/cc) at 35,000 r.p.m. for 48 hours at 20°C in an SW 50 rotor. Four-drop fractions were collected. Densities of the fractions were determined according to the method of Schildkraut *et al.* (104). An aliquot (20 µl) of the fractions was used for radioactivity determination.

O: ^{3}H-A26 DNA

●: ^{14}C-Sp^{-}H12-3 DNA

X: Density.


30
20
10
0
1.5
1.0
0.5
0
1
10
20
30
40
50
TUBE NO.
^{3}H-A26 DNA C.P.M. X 10^{-3}(o——o)
^{14}C-H12-3 DNA C.P.M X 10^{-3} (•----•)
DENSITY (g/cc) (x——x)
1.72
1.71
1.70
1.69
1.68

of 0.19 with respect to $HgCl_2$. The distribution of A26 and Sp^-H12-3 DNA fragments in a Hg-Cs_2SO_4 gradient is shown in Fig. 11. More than 95% of input radioactivity of either ^3H-A26 or ^{14}C-Sp^-H12-3 DNA was recovered in fractions from 11 to 40. The ratios of $^3H/^{14}C$ in most fractions were found to be very similar. However, relatively high ratios were observed in fractions 20, 33 and 36.

In order to determine whether the broad distribution of DNA shown in Fig. 11 really reflected differences in the G + C content, ^{32}P-labelled A26 DNA was sheared and centrifuged by the same procedure. Fractions collected were subjected to nucleotide analysis (65,134) and their G + C contents were determined and shown in Fig. 11. The results showed that the G + C content of A26 DNA fragments of about 1.5×10^6 daltons varied from 34.5 to 52.5%, with a mean and standard error of 44.2 ± 3.6%. These values were very close to those observed with another $\underline{sp^+}$ strain of *B. subtilis* (SB19E) by Yamagishi and Takahashi (in press). The G + C contents of fractions 20,33 and 36 were estimated to be 40.5, 47.0 and 49.0%, respectively.

3. DNA-DNA hybridization

If the DNA fragments found in fragments found in fractions 20, 33 and 36 (Fig. 11) really correspond to the deleted fragments in Sp^-H12-3 chromosome, these fragments

Fig. 11. Distribution of ^{3}H-A26 and ^{14}C-Sp^{-}H12-3 DNA fragments in a Hg-Cs_2SO_4 density gradient. ^{3}H-A26 DNA (50 µg, 5.5 x 10^4 c.p.m./µg) and ^{14}C-Sp^{-}H12-3 DNA (150 µg, 752 c.p.m./µg) were mixed and sheared at 33,000 r.p.m. for 20 minutes in a VirTis homogenizer. Sheared DNA mixture was added to a Hg-Cs_2SO_4 solution with an rf value of 0.19 with respect to $HgCl_2$, and centrifuged at 35,000 r.p.m. for 48 hours at 4°C, in a type 50 titanium rotor. Four-drop fractions were collected in tubes containing 1 ml of 2 x SSC. Two-tenth ml samples from each fraction were used for radioactivity determination. To determine the base composition of DNA fragments, 20 µg of ^{32}P-A26 DNA (2.5 x 10^6 c.p.m./µg) were mixed with 100 µg of phage T4 DNA which was used as carrier. The mixture was sheared and centrifuged as above. The G + C content of ^{3}H-A26 and ^{14}C-Sp^{-}H12-3 DNA fragments was estimated by superimposing the result of the base analysis carried out with ^{32}P-labelled DNA.

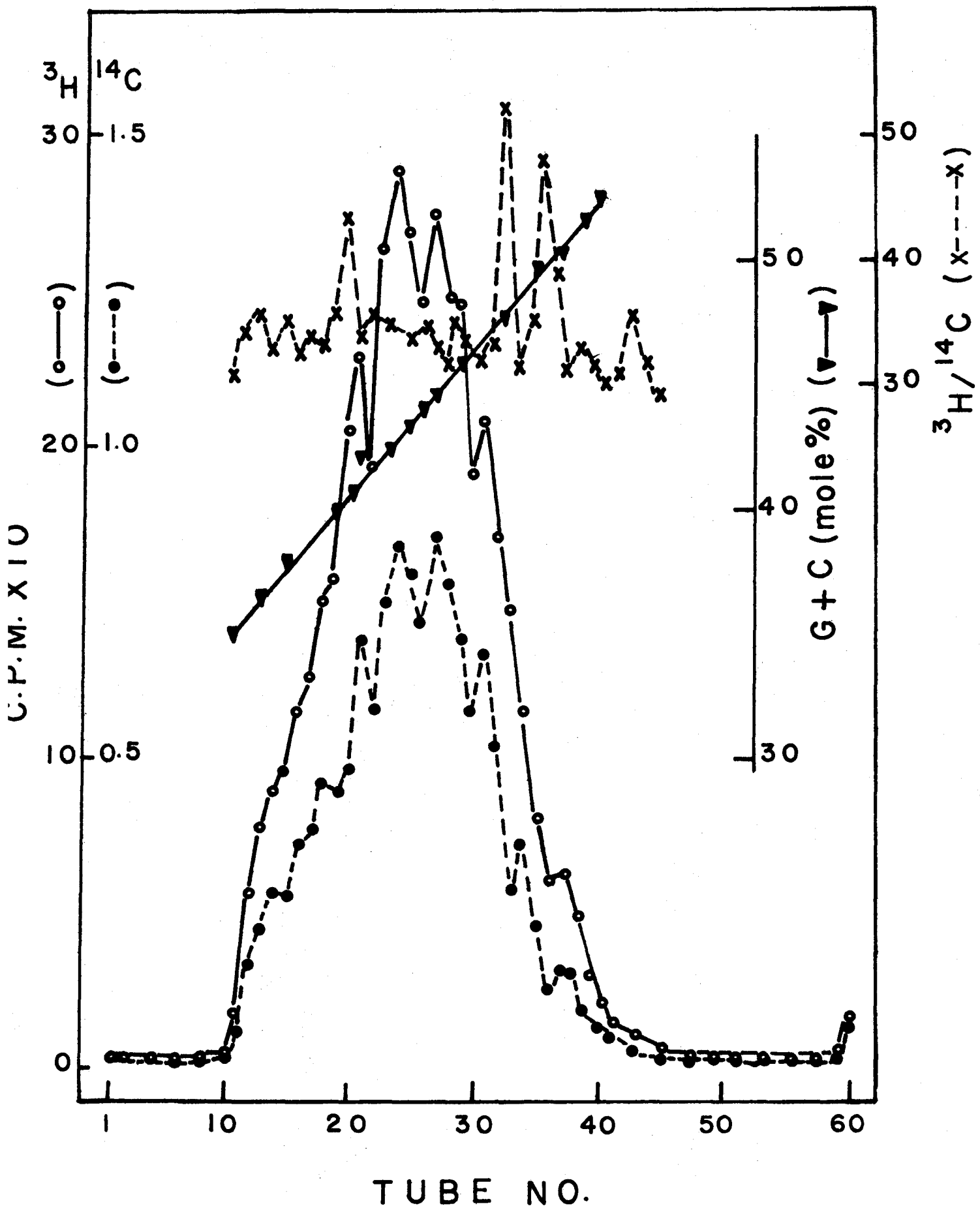
O: ^{3}H-A26 DNA

●: ^{14}C-Sp^{-}H12-3 DNA

X: ratios of $^{3}H/^{14}C$

▼: mole G + C %

The results presented in this figure are from one of five independent experiments, values obtained from which agree within 10%.


^3H ^{14}C
30
1.5
20
1.0
10
0.5
0
C.P.M. X 10
50
40
30
50
40
30
G+C (mole%)
$^3H/^{14}C$ (x---x)
1
10
20
30
40
50
60
TUBE NO.

should hybridize with A26 DNA at much higher efficiencies than those with Sp^-H12-3 DNA. To test this possibility, ^3H-A26 and ^{14}C-Sp^-H12-3 DNA fractions obtained from the Hg-Cs_2SO_4 density gradient centrifugation described in the above section were hybridized with unlabelled A26 or Sp^-H12-3 DNA by the technique of Warnaar and Cohen (128).

Results are expressed as the ratio of the amount of radioactive hybrid formed with A26 DNA to that formed with Sp^-H12-3 DNA. As illustrated in Fig. 12a, fragments of ^3H-A26 DNA in fractions 20, 33 and 36 showed higher ratios than those of other fractions. On the other hand all fractionated ^{14}C-Sp^-H12-3 DNA showed about the same efficiency of hybridization with both Sp^-H12-3 and A26 DNA (Fig. 12b). These results suggest that fractions 20, 33 and 36 of A26 DNA probably contain DNA fragments deleted in Sp^-H12-3.

4. DNA-RNA hybridization

Fractionated DNA fragments obtained by Hg-Cs_2SO_4 density gradient centrifugation were further characterized by the DNA-RNA hybridization technique in which fractionated A26 or Sp^-H12-3 DNA was hybridized with ^3H-A26 t_2 RNA. In order to carry out DNA-RNA hybridization, a relatively large amount of unlabelled A26 DNA and Sp^-H12-3 DNA were sheared and fractionated separately in Hg-Cs_2SO_4 gradients. The distribution of DNA fragments in the gradient is shown

Fig. 12. Analysis of fractionated DNA fragments by DNA-DNA hybridization.

Samples of fractionated ^{3}H-A26 DNA (about 0.12 µg, 5.5 x 10^{4} c.p.m./µg) and ^{14}C-Sp^{-}H12-3 DNA (about 0.26 µg, 752 c.p.m./µg) obtained by Hg-Cs_2SO_4 density gradient centrifugation (see Fig. 11) were hybridized with 100 µg of unlabelled A26 or Sp^{-}H12-3 DNA fixed on nitrocellulose membrane.

C.P.M.(A): the amount of radioactive hybrid formed with unlabelled A26 DNA.

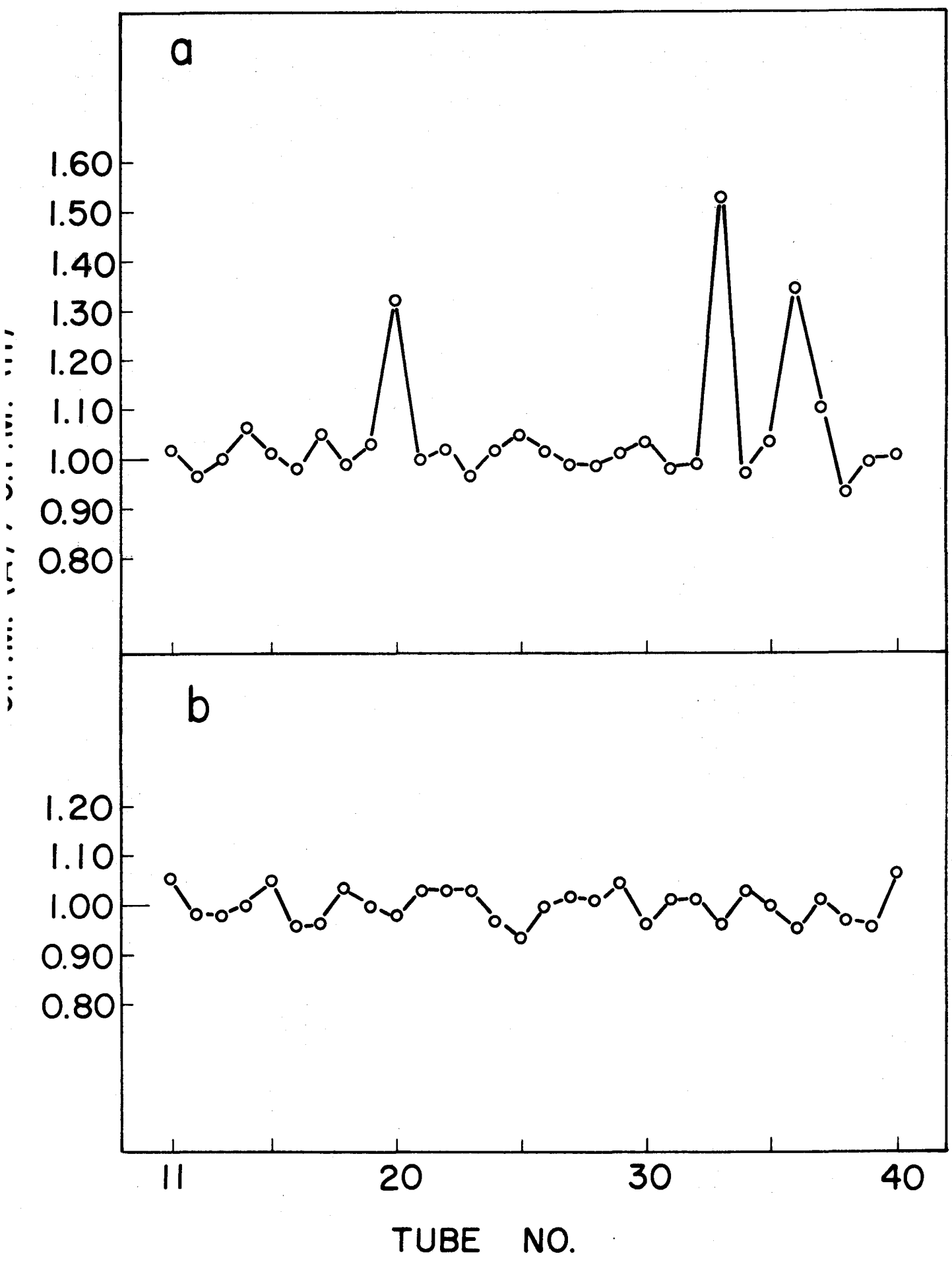
C.P.M.(H): the amount of radioactive hybrid formed with unlabelled Sp^{-}H12-3 DNA.

a - fractionated ^{3}H-A26 DNA

b - fractionated ^{14}C-Sp^{-}H12-3 DNA

Hybridization efficiency (DNA hybridized/DNA input) was in the range of 40 to 60% for both ^{3}H-A26 and ^{14}C-Sp^{-}H12-3 DNA.

Each point represents the average value of six determinations.


a
1.60
1.50
1.40
1.30
1.20
1.10
1.00
0.90
0.80
b
1.20
1.10
1.00
0.90
0.80
11
20
30
40
TUBE NO.

in Fig. 13. Most input A26 or Sp^-H12-3 DNA was recovered in fractions between 10 and 35. Fractionated DNA samples were dialysed against a Tris buffer (0.01 M Tris, 0.5 M KCl, pH 7.3) prior to heat-denaturation and hybridization. Since the amount of DNA available for this experiment was very small, a liquid DNA-RNA hybridization technique described by Yamagishi and Takahashi (136) was employed. Results are expressed as the ratio of the amount of ^{3}H-RNA hybridized with A26 DNA to that hybridized with Sp^-H12-3 DNA.

When ^{3}H-A26 t_2 Gr. I or III RNA was used, about the same amount of RNA hybridized with both A26 DNA and Sp^-H12-3 DNA from all fractions (Figs. 14a and 14c). However, when ^{3}H-A26 t_2 Gr. II RNA was used, the amount of RNA hybridized with A26 DNA was much higher than that hybridized with Sp^-H12-3 DNA in fractions 17 and 31 (Fig. 14b). The G + C content of fractions 17 and 31 was estimated to be about 40 and 49% respectively. Results obtained from experiments described in the previous sections suggest that the G + C content of the DNA segments deleted in Sp^-H12-3 would be 40.5, 47.0 and 49.0%. It appears therefore that the fragment having a G + C content of 47% in A26 DNA may not be transcribed at the stage t_2.

Fig. 13. Distribution of unlabelled A26 and $Sp^{-}H12$-3 DNA in Hg-Cs_2SO_4 density gradient.

Unlabelled A26 DNA (500 μg) or $Sp^{-}H12$-3 DNA (475 μg) was sheared and centrifuged in Hg-Cs_2SO_4 density gradients separately. Four-drop fractions were collected in tubes containing 1 ml of 1 x SSC, and optical densities of the fractions were measured at 260 nm.

The procedure for centrifugation and base analysis was described in the footnote of Fig. 11.

O: A26 DNA

●: $Sp^{-}H12$-3 DNA

▼: mole G + C%

The values of optical density shown in this figure are from one of three independent experiments, values obtained from which agree within 10%.

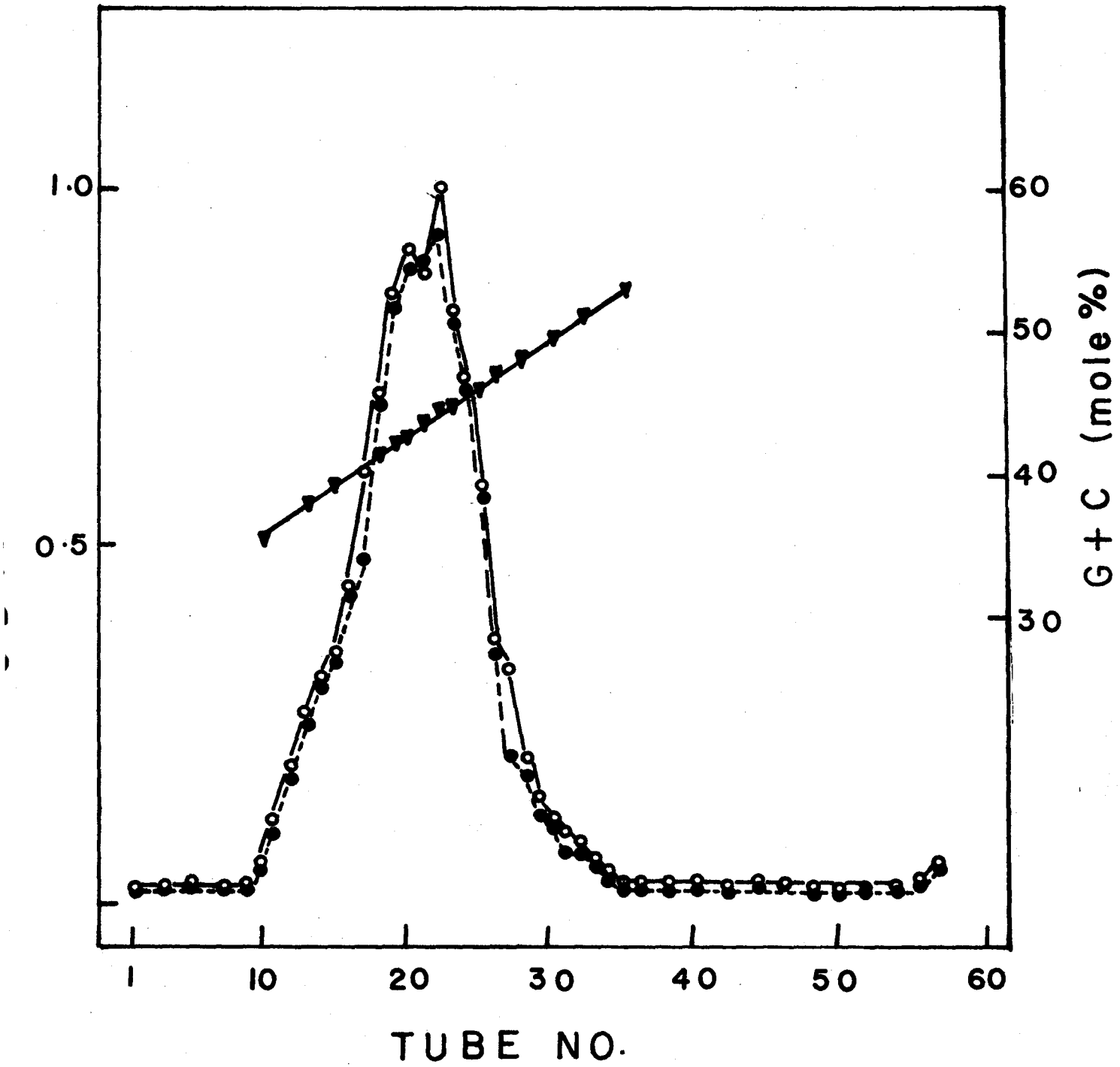

1·0
0·5
60
50
40
30
G+C (mole %)
1
10
20
30
40
50
60
TUBE NO.

Fig. 14. Hybridization between fractionated DNA fragments and pulse-labelled A26 t_2 RNA.

^{3}H-A26 t_2 RNA was fractionated in a linear sucrose gradient as described in Fig. 6. Samples of DNA were fractionated in Hg-Cs_2SO_4 gradients (Fig. 13). Hybridization mixture contained 1 µg of A26 or Sp^-H12-3 DNA and 0.1 µg of ^{3}H-A26 t_2 Gr. I. (120,000 c.p.m./µg), or Gr. II (34,000 c.p.m./µg), or Gr. III (42,500 c.p.m./µg) RNA.

C.P.M.(A): the amount of radioactive hybrid formed with A26 DNA.

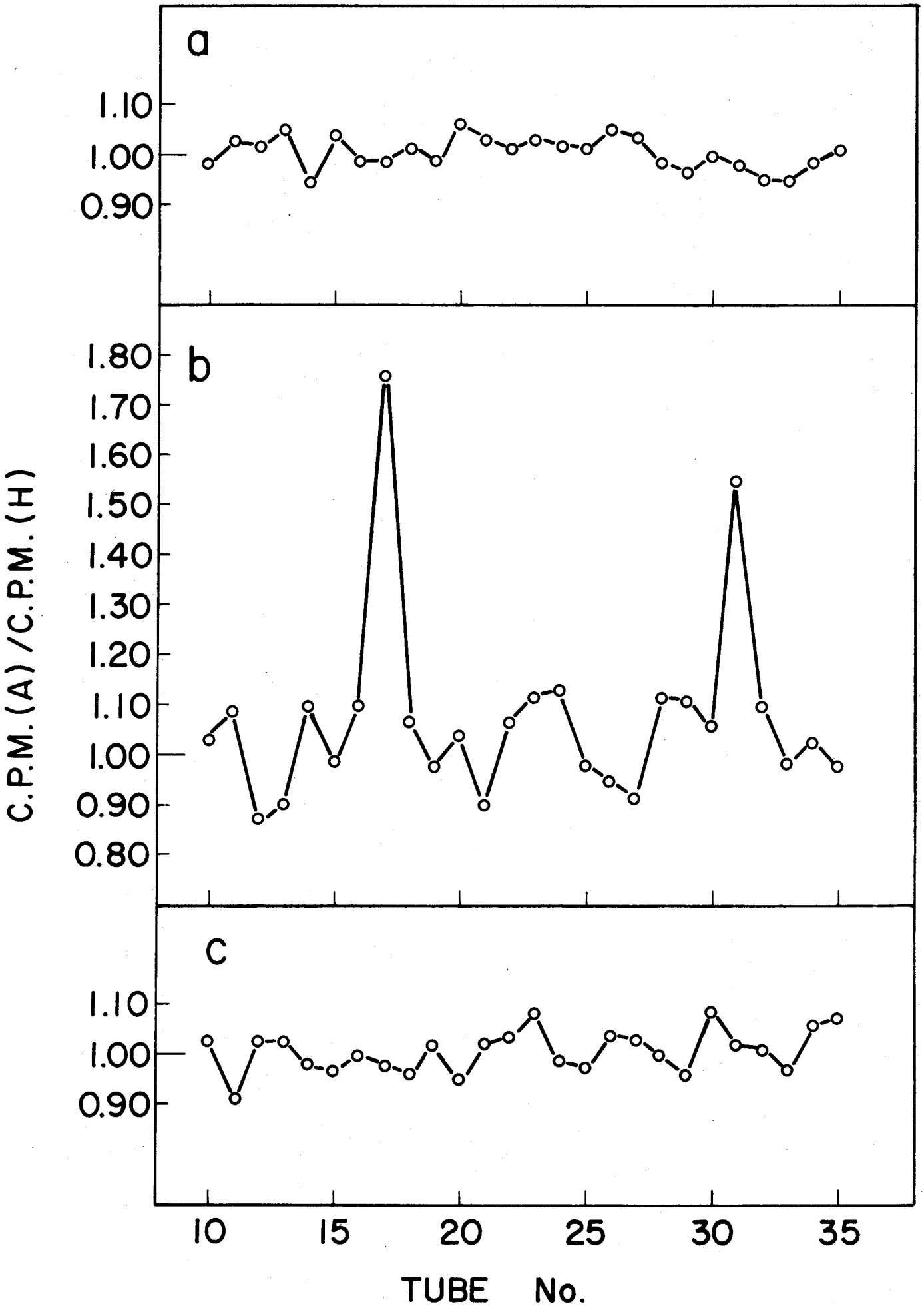
C.P.M.(H): the amount of radioactive hybrid formed with Sp^-H12-3 DNA.

a - ^{3}H-A26 t_2 Gr. I. RNA

b - ^{3}H-A26 t_2 Gr. II RNA

c - ^{3}H-A26 t_2 Gr. III RNA

Hybridization efficiency (RNA hybridized/RNA input) was in the range of 5 to 12%. Each point represents the average value of six determinations.


a
1.10
1.00
0.90
b
1.80
1.70
1.60
1.50
1.40
1.30
1.20
1.10
1.00
0.90
0.80
c
1.10
1.00
0.90
C.P.M. (A) /C.P.M. (H)
10
15
20
25
30
35
TUBE No.

CHAPTER IV

PHYSIOLOGICAL AND BIOCHEMICAL CHANGES IN STRAIN SP^-H12-3

Genetic and biochemical investigations on Sp^-H12-3 carried out previously by Takahashi (122,123,124,136) suggested that deletion in this strain might cover genes which are responsible for regulation of sporulation. Experiments presented in this chapter were conducted in order to determine physiological or biochemical function(s) which may be defective in Sp^-H12-3.

1. Changes in pH and pyruvic acid

Changes in pH value as well as in the accumulation and reutilization of pyruvic acid during sporulation in cultures of Sp^-H12-3 and A26 (sp^+) have been determined. These changes are known to be related to the glyoxylic acid cycle, which is, in turn closely associated with the process of sporulation (24,27,32,78).

Cells of A26 or Sp^-H12-3 were grown in SSM on a 37°C water bath shaker. The pH and the amount of pyruvic acid were measured at intervals.

The pH of the culture fluid started to decrease at 1.5 hours after inoculation and reached a minimal value at t_0 in both A26 and Sp^-H12-3 (Fig. 15). Shortly after

t_0, the pH of both cultures began to rise and the pH value increased steadily during sporulation. After 24 hours of growth, the pH value reached to 8.8 in both strains tested. At this time 85 to 95% of A26 cells were converted to refractile spores and, as a consequence, the optical density as measured by a Klett colorimeter of this culture increased considerably. For Sp^-H12-3, a marked decrease in optical density was observed. Microscopic examination of the culture showed that the decrease in optical density was due to autolysis of the cells.

The amount of pyruvic acid started to increase in both cultures at the same time when the pH began to drop (i.e., about 1.5 hours after inoculation), and reached a maximal value at t_0. Reutilization of pyruvic acid after t_0 was accompanied by a rise in the pH value. Most of it has been utilized at about t_2. Changes in the pH and pyruvic acid were also determined in strains SB19E and Sp^-N2-2, and the results observed (not shown in Fig. 15) were very similar to those described above.

2. Acid-soluble nucleotides

To investigate nucleotides, cells of A26 or Sp^-H12-3 were extracted with 5% TCA. The nucleotide samples, after concentration, was adsorbed on a Dowex-1 column and eluted with a formic acid gradient by the technique of Hurlbert (38). Elution profiles of nucleotides extracted from A26 and

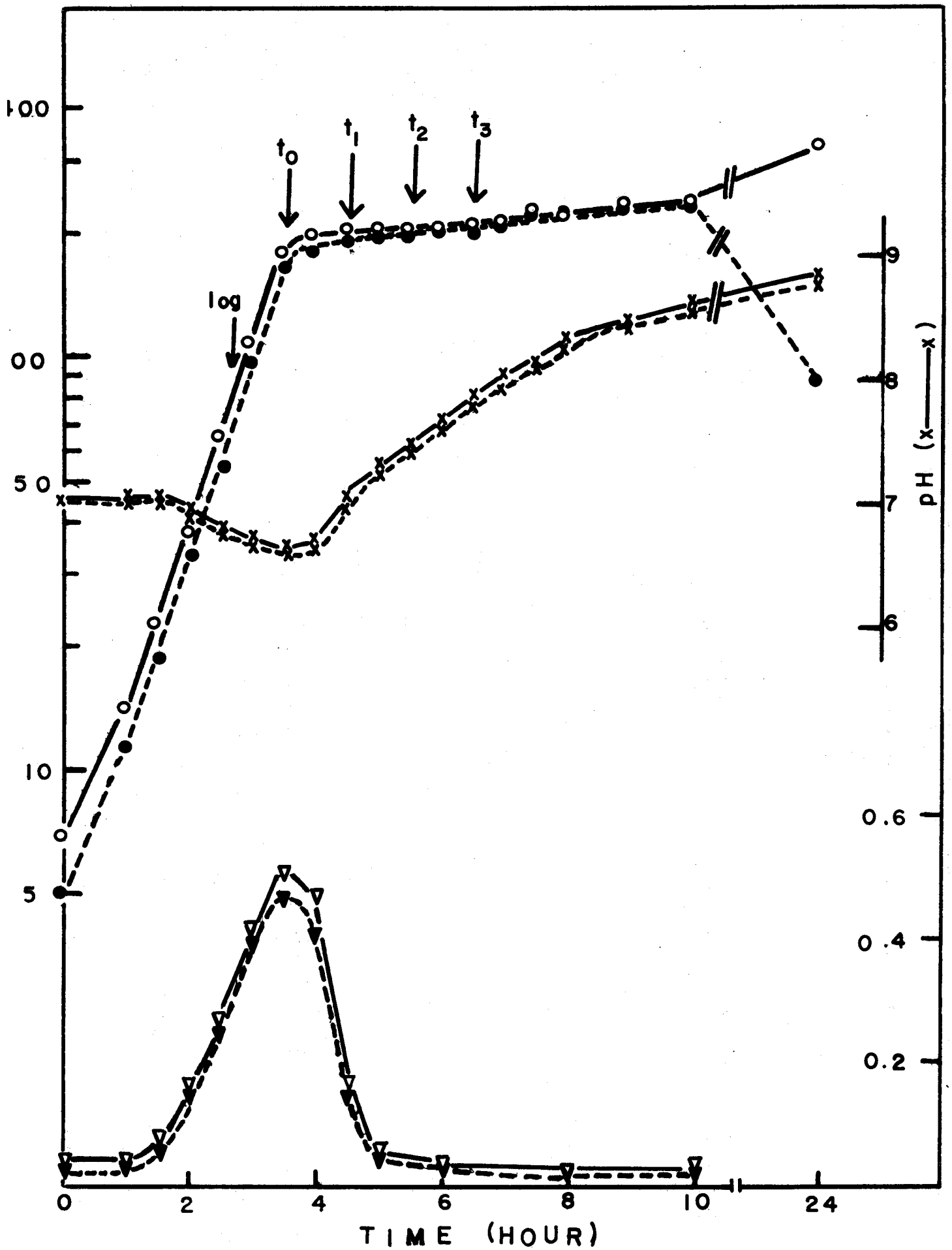
Fig. 15. Changes in pH and pyruvic acid in A26 and $Sp^{-}H12$-3.

Cells were grown in Schaeffer's sporulation medium on a 37°C water bath shaker. The optical density (O), pH (X) and pyruvic acid (∇) were determined.

Various stages of growth are indicated by arrows.

Solid lines - A26

Broken lines - $Sp^{-}H12$-3


100
00
50
10
5
log
t_0
t_1
t_2
t_3
9
8
7
6
pH (x——x)
0.6
0.4
0.2
PYRUVATE (▽——▽) µM/ml
0
2
4
6
8
10
24
TIME (HOUR)

Sp^{-}H12-3 are shown in Figs. 16 (log), 17 (t_0), 18 (t_1), 19 (t_2) and 20 (t_3). Ultraviolet-absorbing materials were identified by measuring their absorption spectra in the wavelength range of 200 to 300 nm and by paper chromatography (110). Most UV-absorbing peaks contained common nucleotides (e.g., AMP, CMP, GDP, UDP, etc.), except three peaks eluted at the tube numbers 118 to 123, 126 to 130 and 133 to 138. They were designated as X_1, X_2 and X_3. Ultraviolet absorption spectra of these three compounds indicated that they might be derivatives of uridine. In order to identify them, the chromatographic behaviour of various uridine-containing compounds on a Dowex-1 column was compared with that of the unknown nucleotides. The relative positions shown in Fig. 21 indicate that nucleotide X_1, X_2 and X_3 might be UDPAG, UDP-Gal and UDPG, respectively. The $R_{adenosine}$ values obtained by a paper chromatographic technique (110) for nucleotides X_1 (0.55), X_2 (0.42), and X_3 (0.44) were very close to those of UDPAG (0.56), UDP-Gal (0.41) and UDPG (0.44). The sugar moieties in these three nucleotides were separated and identified by the techniques of Aminoff *et al.* (2) and Ashwell (5). It was found that the absorption spectrum of the sugar extracted from nucleotide X_1, after treated with dimethylaminobenzaldehyde, was almost identical with that of N-acetylglucosamine (Fig. 22). The sugar liberated by the method of Ashwell (5) from nucleotides X_2 and X_3

Fig. 16. Elution profiles of TCA-soluble nucleotides extracted from A26 and Sp⁻H12-3 in the log-phase.

Cells were grown in Schaeffer's sporulation medium. Acid-soluble nucleotides were extracted with 5% TCA from 10 g (wet weight) of cells in the log-phase of growth. The nucleotides were eluted in a Dowex-1 (formate form) column by the formic acid system of Hurlbert *et al.* (38). Ultraviolet-absorbing materials from fractions eluted in the first 35 tubes were pooled and purified further by the formate system (38). Nucleotides were identified by paper chromatography (110) and from their absorption spectra in the wavelength range of 200 to 300 nm (38).

Both nucleotides CMP and AMP were eluted at two different positions. The first peak contained mainly 5'-nucleotides and the second one 2'- and 3'-nucleotides.

———— A26

---------- Sp⁻H12-3

Results shown in this figure are the average of two determinations.

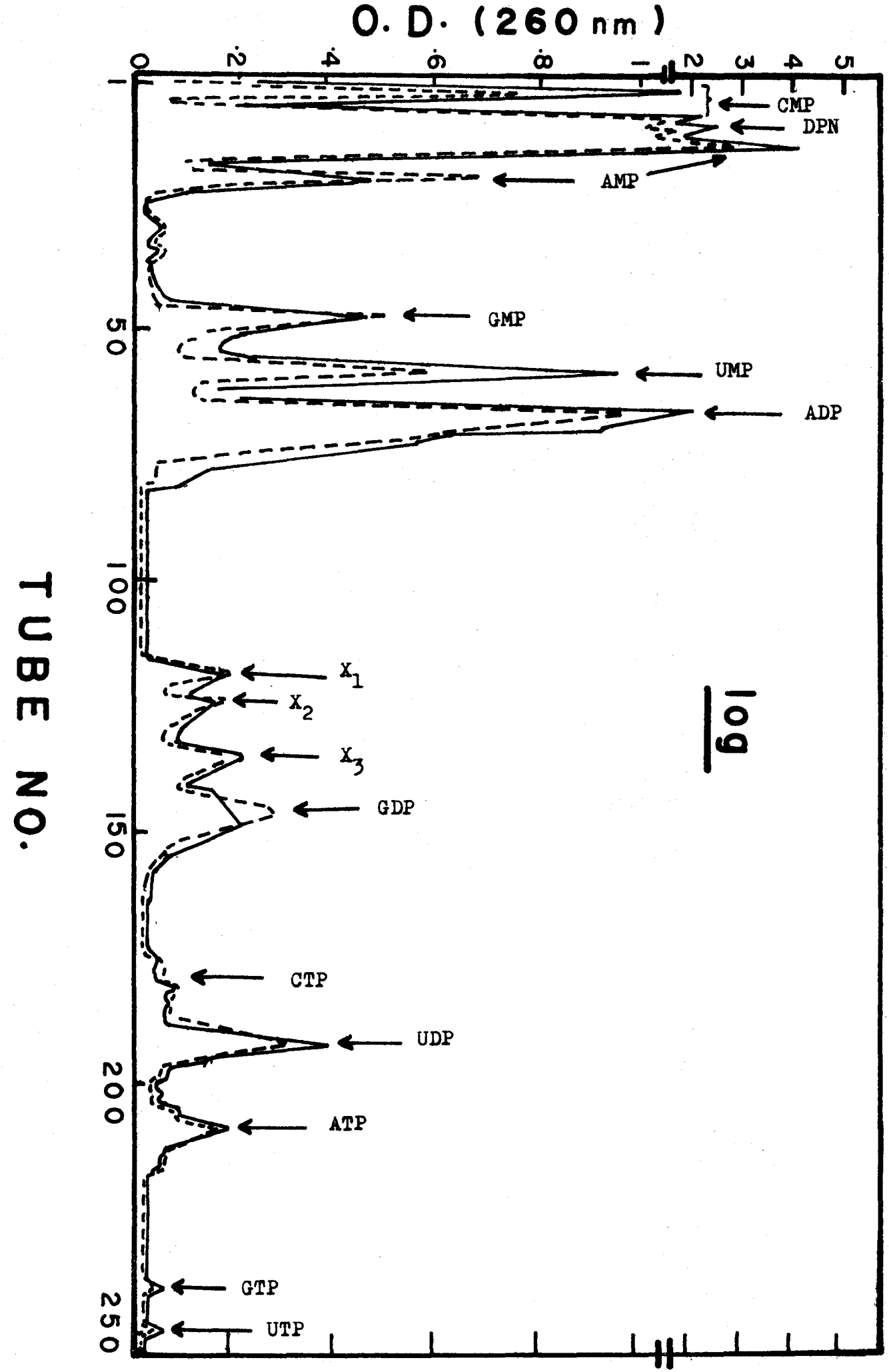

O. D. (260 nm)
0
2
4
6
8
—
2
3
4
5
1
50
100
150
200
250
TUBE NO.
CMP
DPN
AMP
GMP
UMP
ADP
X1
X2
X3
GDP
CTP
UDP
ATP
GTP
UTP
10g

Fig. 17. Elution profiles of TCA-soluble nucleotides extracted from A26 and $Sp^{-}H12-3$ at t_0.

The legend is the same as in Fig. 16, except that nucleotides were extracted at t_0 instead of in the log-phase.

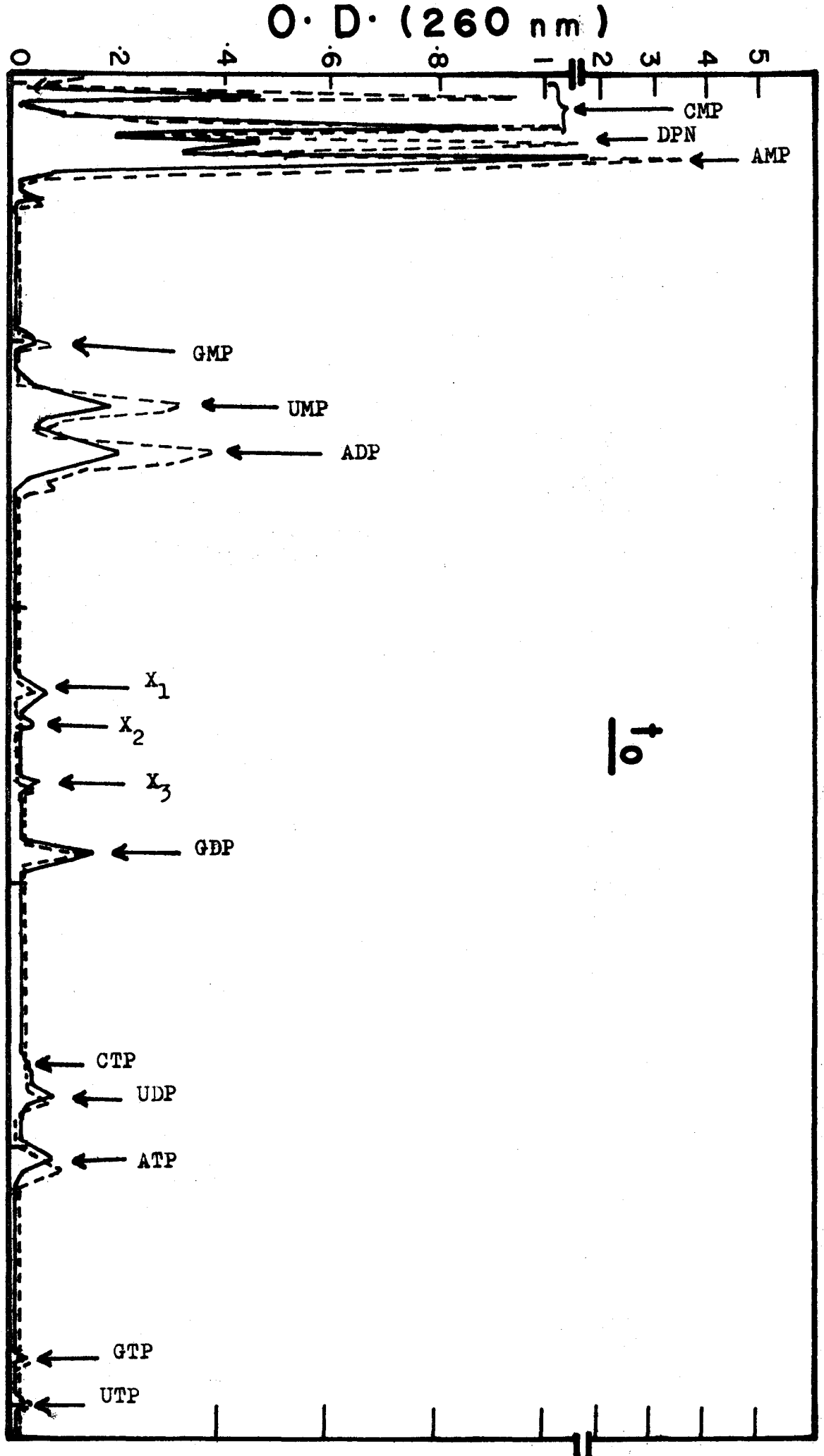

O·D·(260 nm)
0
·2
·4
·6
·8
1
2
3
4
5
CMP
DPN
AMP
GMP
UMP
ADP
X1
X2
X3
GDP
CTP
UDP
ATP
GTP
UTP
1
50
100
150
200
250
TUBE NO.

Fig. 18. Elution profiles of TCA-soluble nucleotides extracted from A26 and Sp^{-}H12-3 at t_1.

The legend is the same as in Fig. 16, except that nucleotides were extracted at t_1 instead of in the log-phase.

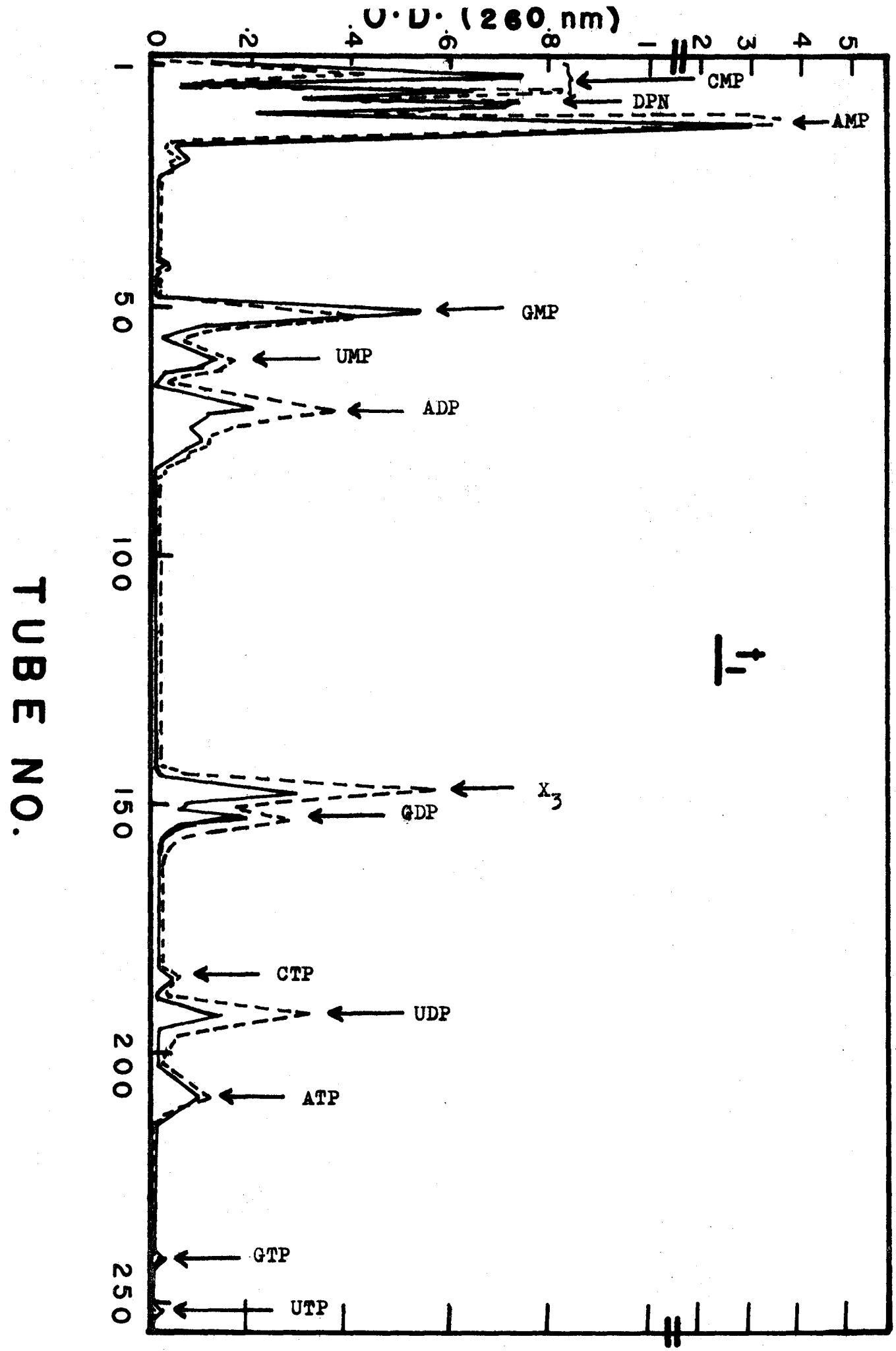

O·D· (260 nm)
0
.2
.4
.6
.8
1
2
3
4
5
CMP
DPN
AMP
GMP
UMP
ADP
X3
GDP
CTP
UDP
ATP
GTP
UTP
1
50
100
150
200
250
TUBE NO.

Fig. 19. Elution profiles of TCA-soluble nucleotides extracted from A26 and $Sp^{-}H12-3$ at t_2.

The legend is the same as in Fig. 16, except that nucleotides were extracted at t_2 instead of in the log-phase.

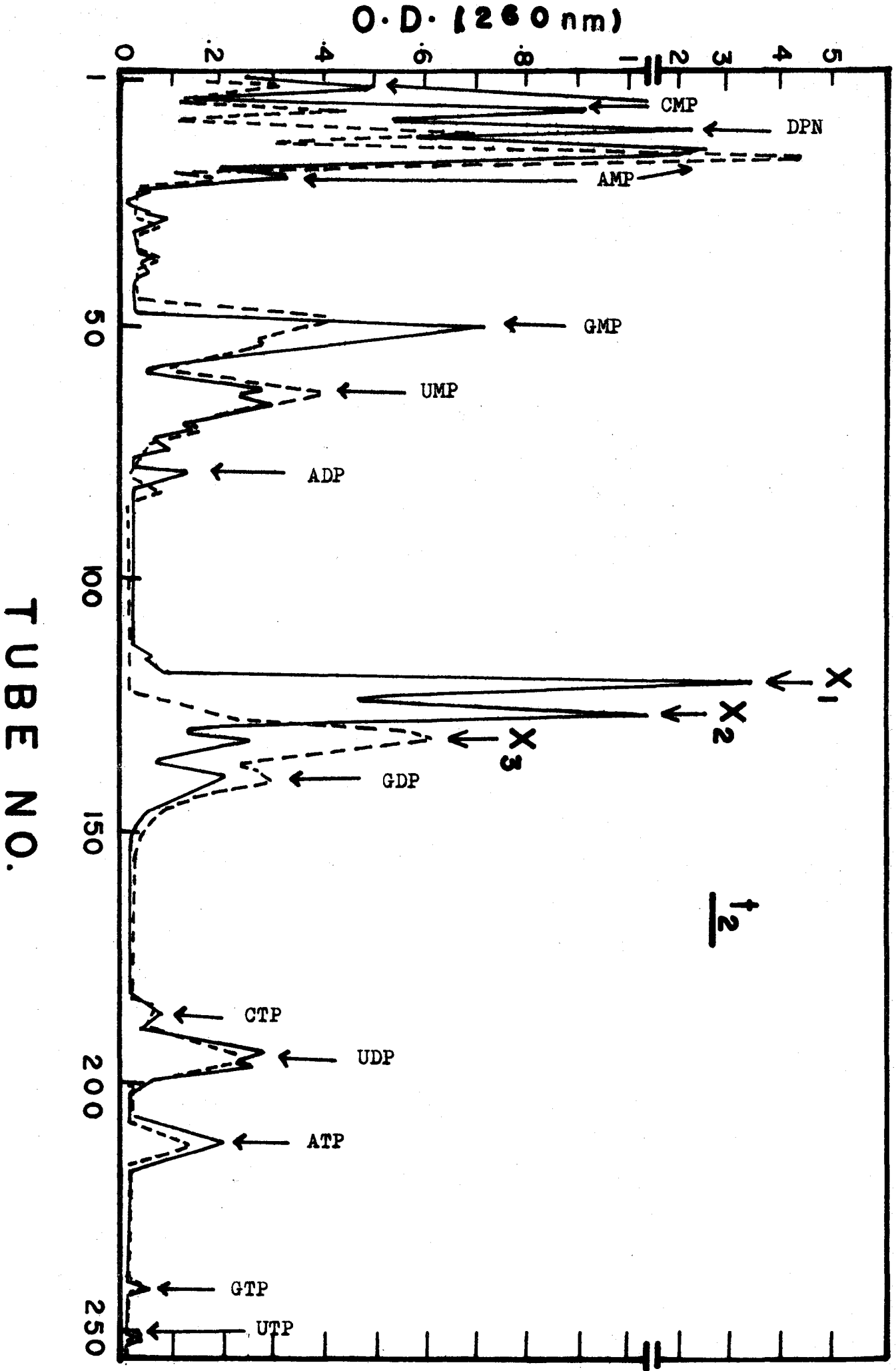

O·D· (260 nm)
0
2
4
6
8
1
2
3
4
5
TUBE NO.
1
50
100
150
200
250
CMP
DPN
AMP
GMP
UMP
ADP
X1
X2
X3
GDP
1/2
CTP
UDP
ATP
GTP
UTP

Fig. 20. Elution profiles of TCA-soluble nucleotides extracted from A26 and $Sp^{-}H12-3$ at t_3.

The legend is the same as in Fig. 16, except that nucleotides were extracted at t_3 instead of in the log-phase.

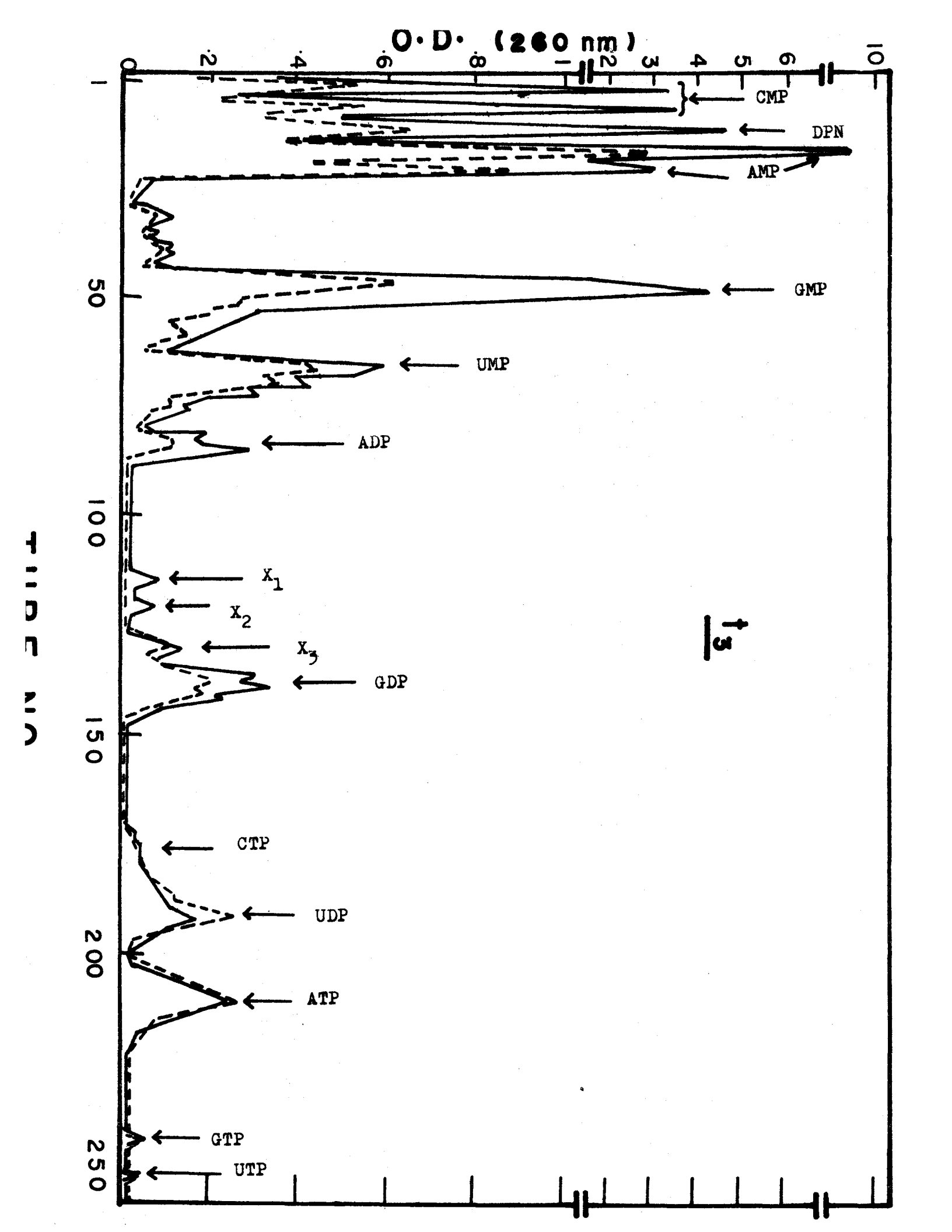

O·D· (260 nm)
0
2
4
6
8
—
2
3
4
5
6
10
1
50
100
150
200
250
TUBE NO.
CMP
DPN
AMP
GMP
UMP
ADP
X1
X2
X3
GDP
CTP
UDP
ATP
GTP
UTP
3

Fig. 21. Relative positions of some uridine-containing nucleotides and their derivatives.

Uridine-containing nucleotides or their derivatives were eluted in a Dowex-1 (formate form) by the same procedure as described in Fig. 16.

UMP - Uridine monophosphate

UDP - Uridine diphosphate

UDPGS - Uridine diphosphoglucosamine

UDPAG - Uridine diphospho-N-acetylglucosamine

UDP-Gal - Uridine diphospho-galactose

UDPG - Uridine diphospho-glucose

UDPGA - Uridine diphospho-glucuronic acid

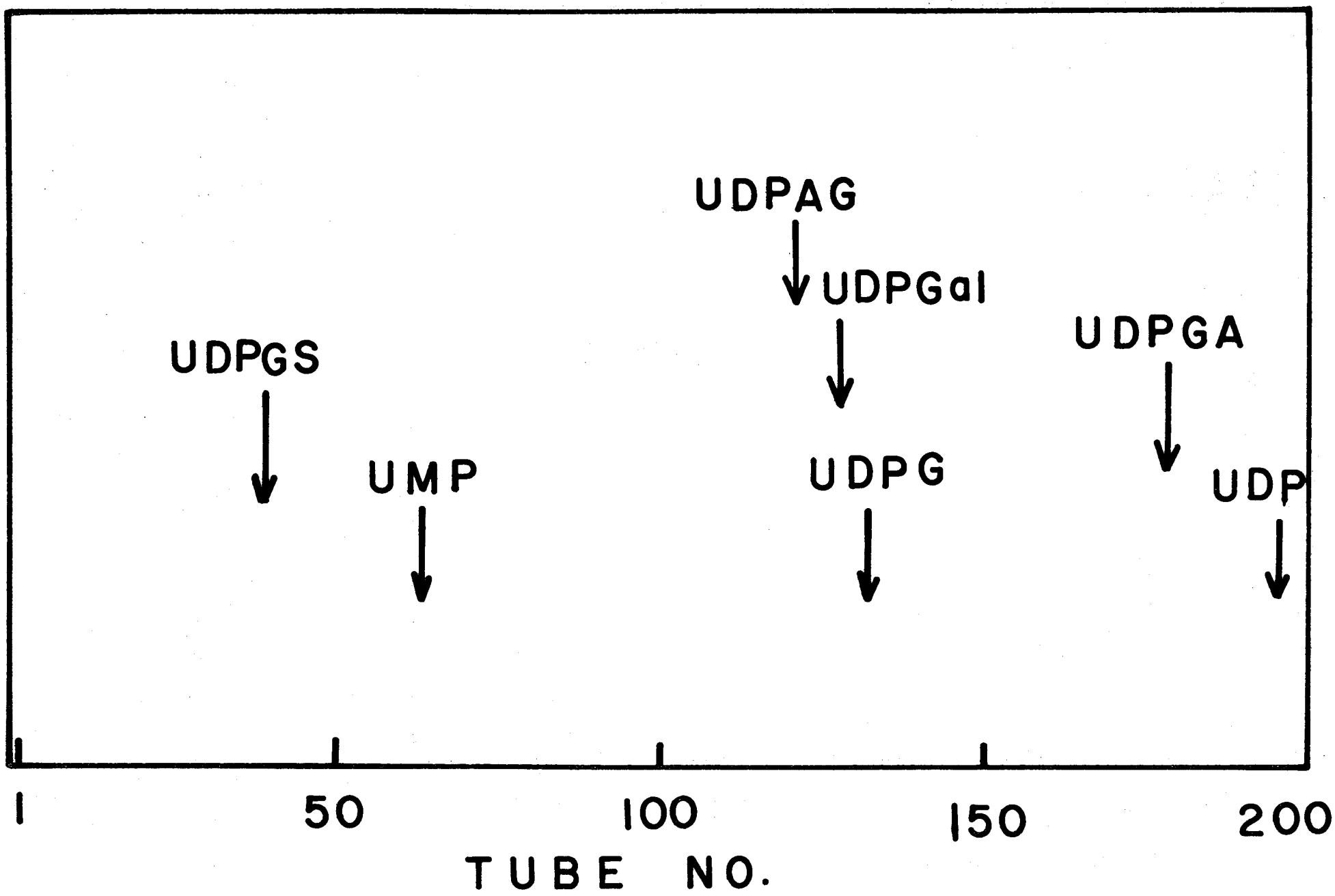
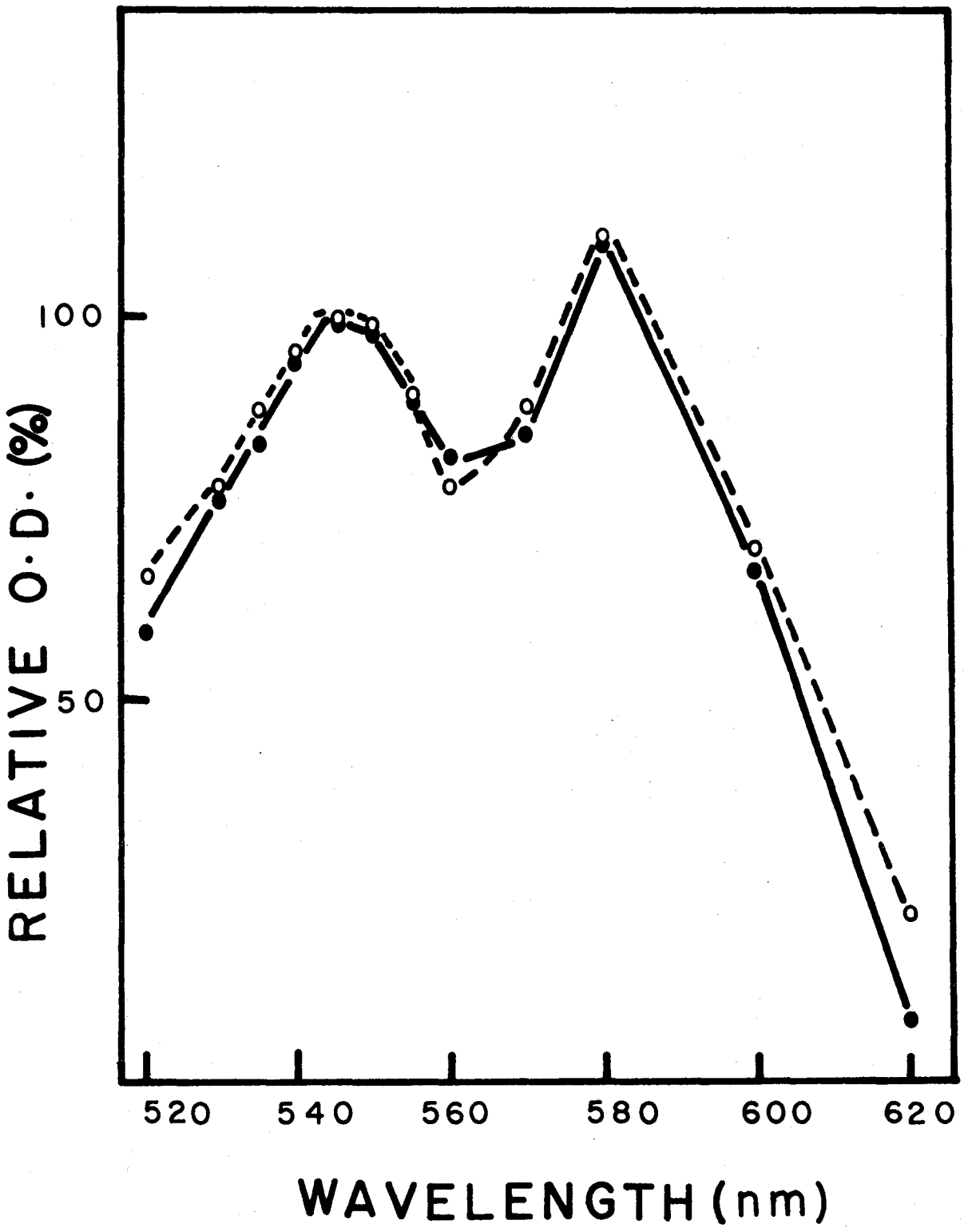

UDPAG
UDPGal
UDPGS
UDPGA
UMP
UDPG
UDP
1
50
100
150
200
TUBE NO.

Fig. 22. Absorption spectrum of N-acetylglucosamine and the sugar moiety isolated from nucleotide X_1 after reaction with dimethylaminobenzaldehyde.

N-acetylglucosamine or the sugar moiety isolated from nucleotide X_1 of A26 t_2 nucleotide sample was treated with dimethylaminobenzaldehyde by the method of Aminoff *et al.* (2). The absorption spectrum of the red-coloured product formed was determined in the wavelength range of 520 to 620 nm.

●: N-acetylglucosamine

O: sugar extracted from nucleotide X_1


100
50
RELATIVE O·D· (%)
520
540
560
580
600
620
WAVELENGTH (nm)

were identified as galactose and glucose from their characteristic colour developed with the cysteine-sulphuric acid reagent. The result of phosphorous determination together with the above data on hexoses revealed a ratio of hexose:phosphorous:uridine to be 1:2:1 (Table I). From the above results nucleotides X_1, X_2 and X_3 can be identified as UDPAG, UDP-Gal and UDPG, respectively.

3. Quantitative determination of TCA-soluble nucleotides

The amounts of nucleotides were determined from their optical densities at pH 1 and molar extinction coefficients (9.9 at 261 nm for U; 13.7 at 280 nm for C; 14.1 at 258 nm for A; and 13.3 at 256 nm for G) (38). Results are shown in **Table** II. It was found that the concentration of nucleoside monophosphates examined increased considerably in A26 between t_1 and t_3, but remained at about the same level in Sp^-H12-3. No marked changes were observed in nucleoside di- and tri- phosphates in both A26 and Sp^-H12-3.

The concentration of DPN which is a cofactor for enzymatic formation of UDP-Gal was lower at presporulation stages (t_0 and t_1) than that in log-phase in A26. At t_3, the concentration of this nucleotide increased to a level which was even higher than that in log-phase. In contrast, in Sp^-H12-3 this nucleotide gradually decreased to a very

Table I

Uridine, phosphorus and hexose contents in nucleotides X_1, X_2 and X_3

Components	Nucleotide							
	X_1[1]		X_2[1]		X_3[1]		X_3[2]	
	µm	ratio	µm	ratio	µm	ratio	µm	ratio
Uridine	0.91	1.00	0.57	1.00	0.54	1.00	1.10	1.00
Total P_i	1.76	1.93	1.20	2.10	1.02	1.90	2.10	1.91
Labile P_i	0.83	0.91	0.55	0.97	0.55	1.02	0.94	0.86
Hexose	0.93	1.02	0.53	0.93	0.48	0.90	1.09	0.99

[1]Nucleotide extracted from A26 cells

[2]Nucleotide extracted from Sp^-H12-3 cells

Table II

Acid-soluble nucleotides in A26 and Sp⁻H12-3[1]

Nucleotide	Amount of nucleotides (μ moles)									
	A26					Sp⁻H12-3				
	log	t_0	t_1	t_2	t_3	log	t_0	t_1	t_2	t_3
AMP	1.69	1.24	1.90	2.83	3.16	1.88	2.03	1.98	2.36	2.30
ADP	0.70	0.20	0.24	0.16	0.23	0.53	0.46	0.30	0.08	0.08
ATP	0.24	0.06	0.07	0.29	0.32	0.22	0.06	0.08	0.18	0.32
GMP	0.37	0.08	0.40	0.88	2.28	0.35	0.09	0.31	0.71	0.74
GDP	0.18	0.11	0.20	0.25	0.30	0.22	0.11	0.28	0.29	0.24
GTP	0.02	0.01	0.01	0.02	0.02	0.02	0.01	0.01	0.01	0.02
CMP	1.26	0.60	0.79	1.20	2.85	0.92	0.90	0.73	0.57	0.86
CDP	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
CTP	0.04	0.02	0.05	0.07	0.05	0.04	0.02	0.05	0.07	0.05
UMP	0.54	0.30	0.41	0.55	1.10	0.38	0.50	0.43	0.45	0.56
UDP	0.34	0.08	0.27	0.40	0.44	0.33	0.08	0.40	0.37	0.51
UTP	0.02	0.01	0.01	0.01	0.02	0.02	0.01	0.01	0.01	0.02
DPN	0.72	0.27	0.17	0.63	1.33	0.67	0.40	0.18	0.17	0.13
UDPAG	0.18	0.08	0.00	1.57	0.07	0.15	0.06	0.00	0.00	0.00
UDP-Gal	0.12	0.03	0.00	0.69	0.06	0.12	0.01	0.00	0.00	0.00
UDPG	0.20	0.04	0.21	0.22	0.18	0.17	0.03	0.57	1.33	0.17
TOTALS	6.62	3.13	4.78	9.77	12.41	5.92	4.77	5.63	5.90	6.20

[1]Nucleotides were extracted from 10 g (wet weight) of cells with 5% TCA. The methods for purification and identification of nucleotides are described in the footnote of Fig. 16. The amounts of nucleotides were determined from their optical densities at pH 1 and molar extinction coefficients (38). All values are the average of two determinations.

low level during presporulation period.

The amount of the three uridine derivatives was about the same in both A26 and Sp⁻H12-3 up to the stage t_1. At t_2, UDPG increased considerably in Sp⁻H12-3, but not in A26. At the same stage, a sudden increase in the amount of UDPAG and UDP-Gal occurred in A26, while no detectable amount of these two compounds was present in Sp⁻H12-3.

Changes in the total amount of nucleic acids and acid-soluble nucleotides were shown in Table II and Fig. 23. As expected, the amount of DNA remained almost constant in both A26 and Sp⁻H12-3 during the presporulation stage. In A26 the total amount of acid-soluble nucleotides increased gradually and correspondingly the amount of RNA decreased during the same period. This change would probably reflect an increased intracellular RNase activity and an active turnover of m-RNA for sporulation. The above change in RNA and nucleotides in A26 was completely absent in Sp⁻H12-3 during presporulation stage.

4. UDP-gal-4-epimerase and galactose-1-phosphate uridyl transferase

As shown in the foregoing section, the concentration of UDPAG and UDP-Gal increases markedly at t_2. Since sporulating cells are forming septum at this stage, it is conceivable that these compounds are associated with the formation of septum. Two enzymes, UDP-gal-4-epimerase and

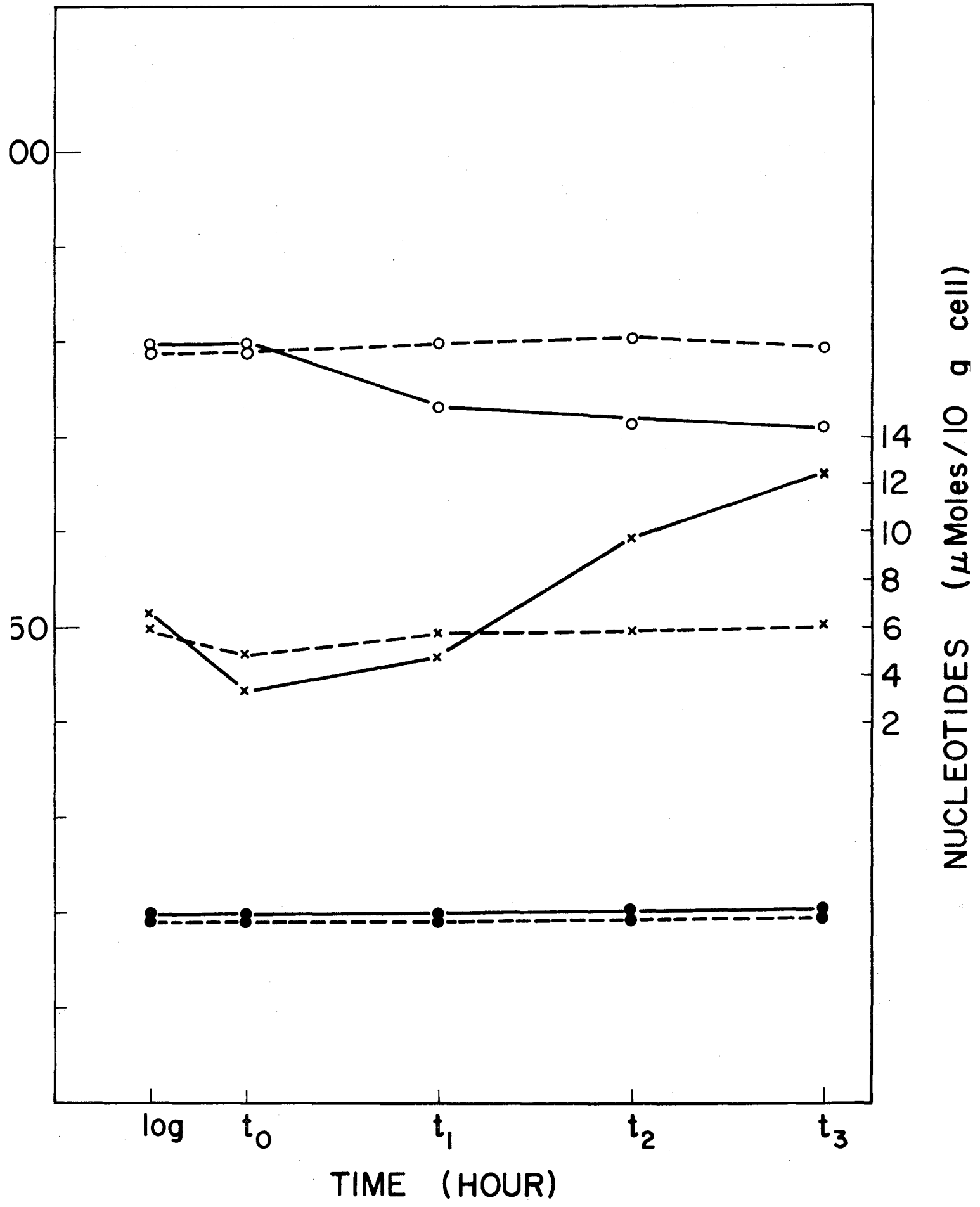
Fig. 23. Changes in nucleic acids and TCA-soluble nucleotides in strains A26 and $Sp^{-}H12$-3 at early stages of sporulation.

Nucleic acids were extracted from 1 g (wet weight) of cells at the stages indicated and the amount of DNA (●) and RNA (O) were determined by using diphenylamine and orcinol tests, respectively. Acid-soluble nucleotides were extracted by 5% TCA, after purification and identification, the total amounts were summed and shown here (X).

The values of nucleic acids are the average of 3 determinations, and those of nucleotides are the average of 2 determinations.

Solid lines - A26

Broken lines - $Sp^{-}H12$-3


00
50
14
12
10
8
6
4
2
NUCLEOTIDES (μMoles/10 g cell)
log
t_0
t_1
t_2
t_3
TIME (HOUR)

galactose-1-phosphate uridyl transferase which are known to catalyse the formation of UDP-Gal from UDPG in A26 ($\underline{sp^+}$), Sp^-H12-3 and Sp^-N2-2 (a stage II mutant) during the presporulation period were investigated. These two enzymes require DPN as cofactor for the reactions. Because suitable substrates were not available, the enzyme which catalyses the formation of UDPAG was not studied.

The specific activity of UDP-gal-4-epimerase increased rapidly after t_0 and reached at a maximal value at t_2 in both A26 and Sp^-N2-2 (Fig. 24). The activity in both strains declined thereafter. Throughtout the experiment the activity of this enzyme in Sp^-H12-3 remained at a rather low level.

The specific activity of galactose-1-phosphate uridyl transferase rose at the end of log-phase and remained almost constant for 2 hours in A26 and Sp^-N2-2. A second increase was observed after t_2 and reached a maximal value at t_3 - $t_{3.5}$ after which time it started to decline (Fig. 25). In Sp^-H12-3, the specific activity of this enzyme was higher than that in A26 or Sp^-N2-2 at stages t_0 and t_1. But it dropped immediately after t_0 and no second increase was detectable.

Fig. 24. Activity of UDP-gal-4-epimerase in A26 $Sp^{-}H12-3$ and $Sp^{-}N2-2$.

X: A26

O: $Sp^{-}H12-3$

●: $Sp^{-}N2-2$

Each point represents the average value of three determinations.

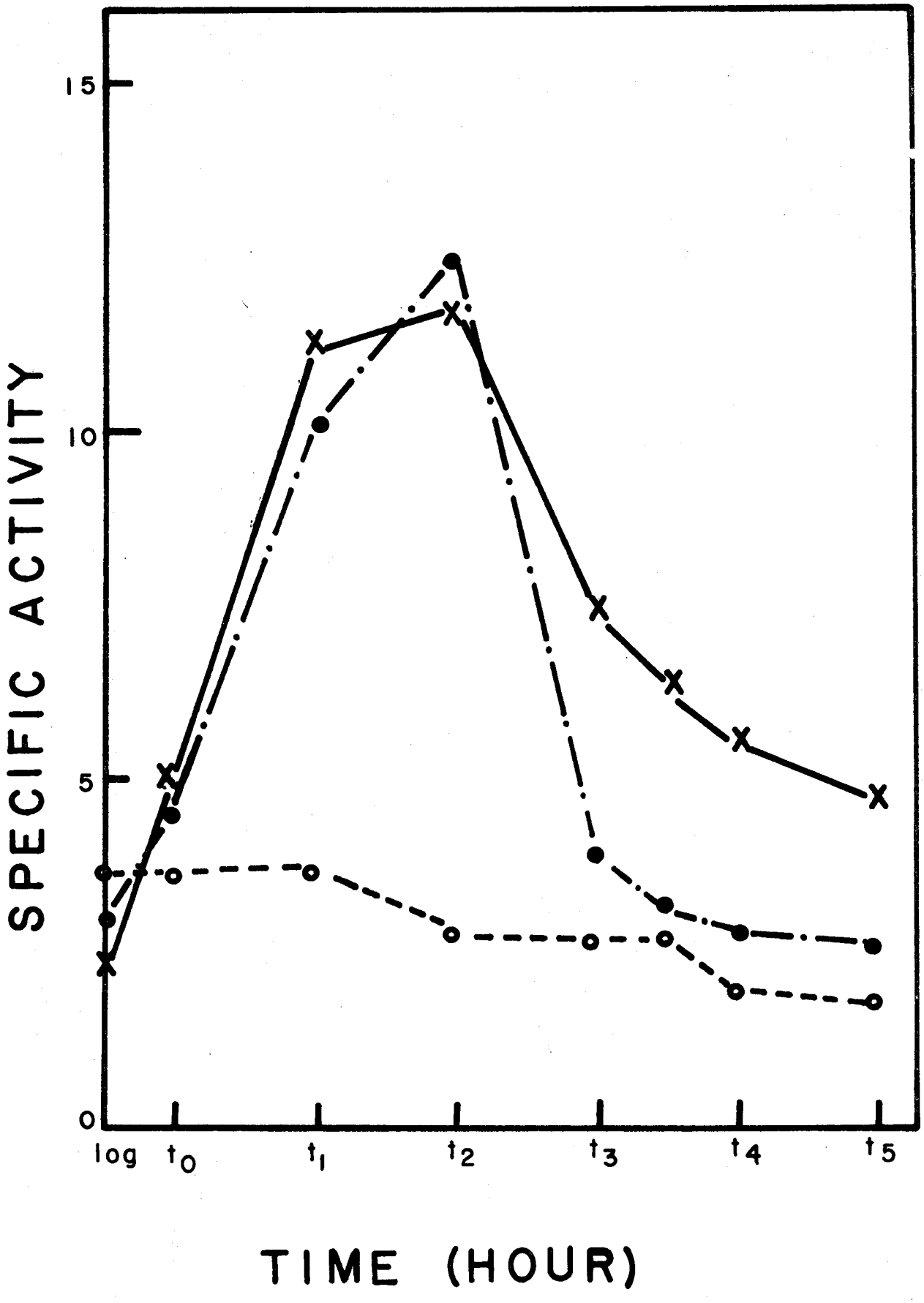

SPECIFIC ACTIVITY
15
10
5
0
log
t_0
t_1
t_2
t_3
t_4
t_5
TIME (HOUR)

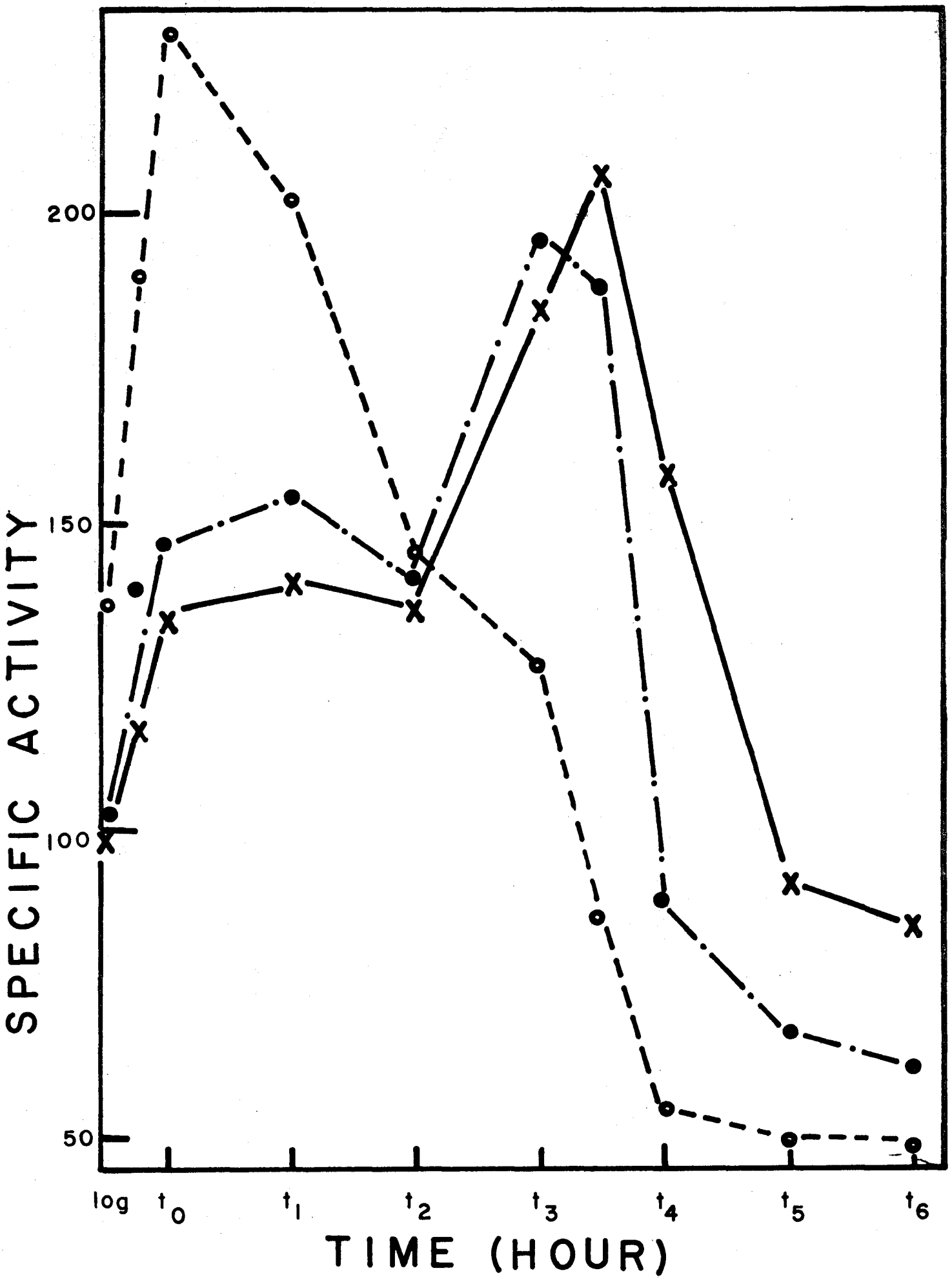
Fig. 25. Activity of galactose-1-phosphate uridyl transferase in A26, $Sp^{-}H12-3$ and $Sp^{-}N2-2$.

X: A26

O: $Sp^{-}H12-3$

●: $Sp^{-}N2-2$

Each point represents the average value of three determinations.


200
150
100
50
SPECIFIC ACTIVITY
log
t_0
t_1
t_2
t_3
t_4
t_5
t_6
TIME (HOUR)

CHAPTER V

DISCUSSION

It is usually assumed that deletion mutants have suffered an actual excision of a fragment of genetic material. Results supporting this assumption have been found in the case of deletions in the rII region of phage T4 (13,14). Weigle (129), by measuring the density difference of phage λ mutants in a CsCl density gradient, has discovered a deletion mutant in which as much as 17% of the genetic material is deleted. A more interesting finding has been recently reported by Westmoreland *et al.* (130), who by using the combination of electron microscopy and DNA-DNA hybridization have physically mapped several deletions in phage λ mutants. However, all these reports mentioned above are carried out with relatively simple bacteriophage systems. In very few cases, if any, has physical demonstration of chromosomal deletion in bacteria been attempted. Deletion in bacteria has been almost exclusively investigated by conventional genetic analyses.

The results obtained with $Sp^{-}H12$-3 suggest the possibility of demonstrating the presence of deletion in the chromosome of bacteria by comparing the distribution of mutant DNA fragments and wild-type DNA fragments in a

density gradient of Hg-Cs_2SO_4, provided that deletion is of a considerable size. Furthermore, by the same technique wild-type DNA fragments which correspond to the deleted portion of mutant chromosome can be isolated and characterized by DNA-mRNA or DNA-DNA hybridization. It has been shown in the present study that three DNA fragments having a G + C content of 40%, 47% and 49% are deleted in Sp^-H12-3 and that only the first and third fragments are transcribed at stage t_2. This finding therefore may be useful not only in studies of sporulation but also in other types of investigations in bacteria.

As for initiation of sporulation, a number of models have been proposed. It is, however, difficult to test the models experimentally, because no biochemical changes have been found to be directly associated with derepression or induction of spore genes. As strain Sp^-H12-3 seems to carry a mutation which blocks very early functions for sporulation, this strain has been chosen to study biochemical changes related to initiation of sporulation.

Changes in pH and pyruvate concentration observed in this mutant are almost identical with those of wild-type bacteria, indicating that the metabolism of pyruvate and possibly acetate is not affected by the mutation. Another metabolic system associated with sporulation is the utilization of poly-β-hydroxybutyric acid which is

known to supply energy and carbon for the process of sporulation. As the utilization of this polymer and sporulation are closely associated, it would be of interest to investigate this compound in Sp^-H12-3.

The search for nucleotides which might be important in the regulation of sporulation in early stages (t_0 - t_1) was not successful. Nevertheless, it has been found that the increase in UDPAG and UDP-Gal in sporulating cells at t_2 is completely absent in Sp^-H12-3. Since this mutant has a block at a stage earlier than t_2 and since septum formation begins in sporulating cells at t_2, these nucleotides may be involved in the formation of the septum.

So far only enzymes concerned in the synthesis of dipicolinic acid are considered to be specifically related to the process of sporulation. Although further studies on the two enzymes (UDP-gal-4-epimerase and galactose-1-phosphate uridyl transferase) which form UDP-Gal from UDPG are necessary to establish a clear relationship between sporulation and this nucleotide, these enzymes appear to be promising candidates to become "spore-specific" enzymes.

It has been found in yeast (45,46,57,131), in *E. coli* (44,47), and in animal cells (3,39,105) that the UDP-Gal generating enzymes are equally active under normal conditions. In contrast, in sporulating *B. subtilis*, these enzyme activities increase sharply during the

presporulation stage and decline rather rapidly thereafter (Figs. 24 and 25). The pattern of changes in enzyme activity is, however, different for the two enzymes. While UDP-gal-4-epimerase shows its maximal activity at t_2, the activity of galactose-1-phosphate uridyl transferase is highest at $t_{3.5}$. This and other observations that the concentration of UDPG (precursor of UDP-Gal) in Sp^-H12-3 is much higher than in sporulating cells and that the concentration of DPN (cofactor for the enzymes) changes markedly during sporulation suggest that the activity of these enzymes may be under the control of a fairly complex system. Further investigations on this aspect should yield useful information on the biochemistry of sporulation.

REFERENCES

1. Abraham, E. P., and H. W. Florey. 1949. Antibiotics from bacteria in the genus *Bacillus*, p. 471-492 and 1533-1541. *In* Antibiotics. Oxford University Press, Oxford, England.

2. Aminoff, D., W. T. J. Morgan, and W. M. Watkins. 1952. Studies in immunochemistry. II. The action of dilute alkali on the N-acetylhexosamines and the specific blood-group mucoids. Biochem. J. 51: 379.

3. Anderson, E. P., H. M. Kalckar, and K. J. Isselbacher. 1957. Defect in uptake of galactose-1-phosphate into liver nucleotides in congenital galactosemia. Science 125: 113.

4. Aronson, A. I. 1965. Characterization of messenger RNA in sporulating *Bacillus cereus*. J. Mol. Biol. 11: 516.

5. Ashwell, G. 1957. Colorimetric analysis of sugars, p. 73. *In* S. P. Colowick and N. O. Kaplan (eds.), Methods in Enzymology III. Academic Press Inc., Publisher, New York.

6. Aubert, J. P., J. Millet, E. Pineau, and G. Milhaud. 1961. L'acide N-succinyl-L(+) glutamique chez *Bacillus megaterium* en voie de sporulation. Biochim. Biophys. Acta 51: 529.

7. Balassa, G. 1964. Quantitative regulation of RNA synthesis during sporulation of *Bacillus subtilis*. Biochem. Biophys. Res. Commun. 15: 236.

8. Balassa, G. 1966. Synthese et fonction des RNA messagers au cours de la sporulation de *Bacillus subtilis*. Ann. Inst. Pasteur 110: 175.

9. Balassa, G. 1969. Biochemical genetics of bacterial sporulation. I. Unidirectional pleiotrophic interactions among genes controlling sporulation in *Bacillus subtilis*. Mol. Gen. Genetics 104: 73.

10. Balassa, G., H. Ionesco, and P. Schaeffer. 1963. Preuve genetique d'une relation entre la production d'un antibiotique par *Bacillus subtilis* et sa sporulation. C. R. Acad. Sci. (Paris) 257: 986.

11. Bayen, H., C. Frehel, A. Ryter, and M. Sebald. 1967. Etude cytologique de la sporulation chez *Clostridium histolyticum*: souche sporogene et mutants de sporulation. Ann. Inst. Pasteur 113: 163.

12. Beall, F. A., M. J. Taylor, and C. B. Thorne. 1962. Rapid lethal effect in rats of a third component formed upon fractionating the toxin of *Bacillus anthracis*. J. Bacteriol. 83: 1274.

13. Benzer, S. 1955. Fine structure of a genetic region in bacteriophage. Proc. Natl. Acad. Sci. U.S. 41: 344.

14. Benzer, S. 1957. The elementary units of heredity, p. 70. *In* W. D. McElroy and B. Glass (eds.), The Chemical Basis of Heredity. Johns Hopkins Press, Baltimore, N.Y.

15. Bernlohr, R. W. 1964. Postlogarithmic phase metabolism of sporulating microorganisms. I. Protease of *Bacillus licheniformis*. J. Biol. Chem. 239: 538.

16. Breitman, T. R. 1963. The feedback inhibition of thymidine kinase. Biochim. Biophys. Acta 67: 153.

17. Burton, K. 1956. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J. 62: 315.

18. Cabib, E., L. F. Leloir, and C. E. Cardini. 1953. Uridine diphosphate acetylglucosamine. J. Biol. Chem. 203: 1055.

19. Canfield, R. E., and J. Szulmajster. 1964. Time of synthesis of deoxyribonucleic acid and protein in spores of *B. subtilis*. Nature 203: 596.

20. Chen, P. S., T. Y. Toribara, and H. Warner. 1956. Microdetermination of phosphorus. Anal. Chem. 28: 1756.

21. Cohn, W. E. 1951. Some results of the application of ion exchange chromatography of nucleic acid chemistry. J. Cellular Comp. Physiol. 38: 21.

22. Coleman, G. 1967. Studies on the regulation of extracellular enzyme formation by *Bacillus subtilis*. J. Gen. Microbiol. 49: 421.

23. Davis, R. 1963. Microbial extracellular enzymes, their uses and some factors affecting their formation, p. 68. *In* C. Rainbow and A. H. Rose (eds.), Biochemistry of Industrial Microorganisms. Academic Press, Inc., N.Y.

24. Dawes, I. W., and J. Mandelstam. 1970. Sporulation of *Bacillus subtilis* in continuous culture. J. Bacteriol. 103: 529.

25. Demerec, M., E. A. Adelberg, A. J. Clark, and P. E. Hartman. 1966. A proposal for a uniform nomenclature in bacterial genetics. Genetics 54: 61.

26. Doi, R. H., and R. T. Igarashi. 1964. Genetic transcription during morphogenesis. Proc. Natl. Acad. Sci. U.S. 52: 755.

27. Freese, E., and P. Fortnagel. 1967. Analysis of sporulation mutants. I. Response of uracil incorporation to carbon sources and other mutant properties. J. Bacteriol. 94: 1957.

28. Gillespie, D., and S. Spiegelman. 1965. A quantitative assay for DNA-RNA hybrids with DNA immobilized on a membrane. J. Mol. Biol. 12: 829.

29. Goldenbaum, P. E., and W. J. Dobrogosz. 1968. The effect of 3',5'-AMP on catabolite repression of β-galactosidase synthesis in *Escherichia coli*. Biochem. Biophys. Res. Commun. 33: 828.

30. Greenberg, R. A., and H. O. Halvorson. 1955. Studies on an autolytic substance produced by an aerobic spore forming bacterium. J. Bacteriol. 69: 45.

31. Gunsalus, I. C. 1955. Extraction of enzymes from microorganisms (bacteria and yeast), p. 51. *In* C. P. Colowick and N. O. Kaplan (eds.), Methods in Enzymology I. Academic Press, Inc., N.Y.

32. Halvorson, H. O. 1962. Physiology of sporulation, p. 223. *In* I. C. Gunsalus and R. Y. Stanier (eds.), The Bacteria IV. Academic Press, Inc., N.Y.

33. Halvorson, H. O. 1965. Sequential expression of biochemical events during intracellular differentiation. Symp. Soc. Gen. Microbiol. 15: 343.

34. Hashimoto, T., S. H. Black, and P. Gerhardt. 1960. Development of fine structure, thermostability and dipicolinate during sporogenesis in *Bacillus*. Can. J. Microbiol. 6: 203.

35. Hershey, A. D., E. Burgi, and L. Ingraham. 1962. Sedimentation coefficient and fragility under hydrodynamic shear as measures of molecular weight of the DNA of phage T5. Biophys. J. 2: 423.

36. Hoch, J. A., and J. Spizizen. 1969. Genetic control of some early events in sporulation of *Bacillus subtilis* 168, p. 112. *In* L. L. Campbell (ed.), Spores IV. American Society for Microbiol., Urbana, Illinois.

37. Holst, E. C., and A. P. Sturtevant. 1940. Relation of proteolytic enzymes to phase of life of *Bacillus larvae*. J. Bacteriol. 40: 723.

38. Hurlbert, R. B., H. Schwitz, A. F. Brumm, and V. R. Potter. 1954. Nucleotide metabolism. II. Chromatographic separation of acid soluble nucleotides. J. Biol. Chem. 209: 23.

39. Isselbacher, K. J., E. P. Anderson, K. Kurahashi, and H. M. Kalckar. 1956. Congenital galactosemia, a single enzymatic block in galactose metabolism. Science 123: 635.

40. Itchinska, E. 1960. Some physiological peculiarities of asporogenous mutants of *Bacilli*. Microbiology (U.S.S.R.) English Transl. 29: 195.

41. IUPAC-IUB Combined Commission on Biochemical Nomenclature, abbreviation and symbols for chemical names of special interest in biological chemistry. 1966. J. Biol. Chem. 241: 527.

42. Ives, D. H., P. A. Morse, Jr., and Van R. Potter. 1963. Feedback inhibition of thymidine kinase by thymidine triphosphate. J. Biol. Chem. 238: 1467.

43. Johnson, C. E., and P. P. Bonventre. 1967. Lethal toxin of *Bacillus cereus*. I. Relationship and nature of toxin, hemolysin, and phospholipase. J. Bacteriol. 94: 306.

44. Kalckar, H. M. 1957. Biochemical mutations in man and microorganisms. Science 125: 108.

45. Kalckar, H. M., B. Braganca, and A. Munch-Petersen. 1953. Uridyl transferases and the formation of uridine diphosphogalactose. Nature 172: 1038.

46. Kalckar, H. M., E. P. Anderson, and K. J. Isselbacher. 1956. Galactosemia, a congenital defect in a nucleotide transferase. Biochim. Biophys. Acta 20: 262.

47. Kalckar, H. M., and E. S. Maxwell. 1956. Some considerations concerning the nature of the enzymic galactose-glucose conversion. Biochim. Biophys. Acta 22: 588.

48. Kerr, I. M., J. R. Chien, and I. R. Lehman. 1967. Exonucleolytic degradation of high molecular weight DNA and RNA to nucleoside 3'-phosphates by a nuclease from *Bacillus subtilis*. J. Biol. Chem. 242: 2700.

49. Kissane, J. M., and E. Robins. 1958. The fluorometric measurement of deoxyribonucleic acid in animal tissues with special reference to the central nervous system. J. Biol. Chem. 233: 184.

50. Klouven, H. M. 1962. The separation of nucleotides from acid-soluble tissue extracts by high voltage paper electrophoresis. J. Chromatog. 7: 216.

51. Koch, A. 1888. Über Morphologie und Entwichlungsgeschichte einigen endosporer Bacterienformen. Bot. Z. 46: 241.

52. Kornberg, A., J. A. Spudich, D. L. Nelson, and M. P. Deutscher. 1968. Origin of proteins in sporulation. Ann. Rev. Biochem. 37: 51.

53. Kuo, J. F., and P. Greengard. 1970. Cyclic nucleotide-dependent protein synthesis. VI. Isolation and partial purification of a protein kinase activated by guanosine 3',5'-monophosphate. J. Biol. Chem. 245: 2493.

54. Kuriki, Y., and A. Kaji. 1968. Factor- and guanosine 5'-triphosphate-dependent release of deacylated transfer RNA from 70 S ribosomes. Proc. Natl. Acad. Sci. U.S. 61: 1399.

55. Larsson, A., and P. Reichard. 1966. Enzymatic synthesis of deoxyribonucleotides. IX. Allosteric effects in the reduction of pyrimidine ribonucleotides by the ribonucleoside diphosphate reductase system of *Escherichia coli*. J. Biol. Chem. 241: 2533.

56. Leitzmann, C., and R. W. Bernlohr. 1965. Changes in the nucleotide pool of *Bacillus licheniformis* during sporulation. J. Bacteriol. 89: 1506.

57. Leloir, L. F. 1951. The enzymatic transformation of uridine diphosphate glucose into a galactose derivative. Arch. Biochem. 33: 186.

58. Levisohn, S., and A. I. Aronson. 1967. Regulation of extracellular protease in *Bacillus cereus*. J. Bacteriol. 93: 1023.

59. Losick, R., and A. L. Sonenshein. 1969. Change in the template specificity of RNA polymerase during sporulation of *Bacillus subtilis*. Nature 224: 35.

60. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randell. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265.

61. Lundgren, D. G., D. F. Karp, and D. R. Lang. 1969. Structure-function relationships in bacterial sporulation, p. 20. *In* L. L. Campbell (ed.), Spores IV. American Society for Microbiol., Urbana, Illinois.

62. Makman, R. S., and E. W. Sutherland. 1965. Adenosine 3',5'-phosphate in *Escherichia coli*. J. Biol. Chem. 240: 1309.

63. Mandell, J. D., and A. D. Hershey. 1960. A fraction column for the analysis of nucleic acids. Anal. Biochem. 1: 66.

64. Mandelstam, J. 1967. End product repression and the regulation of degradation enzymes, p. 351. *In* V. V. Koningsberger and L. Bosch (eds.), Regulation of nucleic acid and protein biosynthesis. Elsevier Publishing Co., Amsterdam.

65. Markham, R., and J. D. Smith. 1952. The structure of ribonucleic acids. 1. Cyclic nucleotides produced by ribonuclease and by alkaline hydrolysis. Biochem. J. 52: 552.

66. Marmur, J. 1961. A procedure for the isolation of deoxyribonucleic acid from microorganisms. J. Mol. Biol. 3: 208.

67. Maxwell, E., S. K. Kurahashi, and H. M. Kalckar. 1962. Enzymes of the Leloir pathway. I. Quantitative determination of enzymatic activity in broken cell preparations, P. 174. *In* S. P. Colowick and N. O. Kaplan (Eds.), Methods in Enzymology V. Academic Press, Inc., N.Y.

68. May, B. K., and W. H. Elliott. 1968. Characteristics of extracellular protease formation by *Bacillus subtilis* and its control by amino acid repression. Biochim. Biophys. Acta 157: 607.

69. Majbaum, W. 1939. Über die Bestimmung kleiner Pentosemengen insbesondere in Derivaten der Adenylsäure. Z. Physiol. Chem. Hoppe-Seyler's 258: 117.

70. Michel, J. F. 1966. L'activite proteolytique du milieu de culture au cours de la croissance et de la sporulation de *Bacillus subtilis*. Ann. Inst. Pasteur 111: 14.

71. Monro, R. E. 1961. Protein turnover and the formation of protein inclusions during sporulation of *Bacillus thuringiensis*. Biochem. J. 81: 225.

72. Murrell, W. G. 1967. The biochemistry of the bacterial endospore. Advan. in Microbiol. Physiol. 1: 133.

73. Nakai, M. Z. Minami, T. Yamazaki, and A. Tsugita. 1965. Studies on nucleases of a strain of *Bacillus subtilis*. J. Biochem. (Tokyo) 57: 96.

74. Nakata, H. M., and H. O. Halvorson. 1960. Biochemical changes occurring during growth and sporulation of *Bacillus cereus*. J. Bacteriol. 80: 801.

75. Nandi, V. S., J. C. Wong, and N. Davidson. 1965. Separation of deoxyribonucleic acid by Hg (II) binding and Cs_2SO_4 density gradient centrifugation. Biochem. 4: 1687.

76. Neuhard, J. 1966. Studies on the acid-soluble nucleotide pool in thymine-requiring mutants of *Escherichia coli* during thymine starvation. III. On the regulation of the deoxyadenosine and deoxycytidine triphosphate pools of *Escherichia coli*. Biochim. Biophys. Acta 129: 104.

77. Neuhard, J., and A. Munch-Peterson. 1966. Studies on the acid-soluble nucleotide pool in thymine-requiring mutants of *Escherichia coli* during thymine starvation. II. Changes in the amounts of deoxycytidine triphosphate and deoxyadenosine triphosphate in *Escherichia coli* 15 $T^-A^-U^-$. Biochim. Biophys. Acta 114: 61.

78. Nishimura, S., and M. Nomura. 1959. Ribonuclease of *Bacillus subtilis*. J. Biochem. (Tokyo) 46: 161.

79. Nomura, M., J. Hosoda, and H. Yoshikawa. 1958. Studies on amylase formation by *Bacillus subtilis*. VI. The mechanism of amylase excretion and cellular structure of *Bacillus subtilis*. J. Biochem. (Tokyo) 45: 734.

80. Norris, J. R. 1957. A bacteriolytic principle associated with cultures of *Bacillus cereus*. J. Gen. Microbiol. 16: 1.

81. Nygaard, A. P., and B. D. Hall. 1964. Formation and properties of RNA-DNA complexes. J. Mol. Biol. 9: 125.

82. Okazaki, R., and A. Kornberg. 1964. Deoxythymidine kinase of *Escherichia coli*. II. Kinetics and feedback control. J. Biol. Chem. 239: 275.

83. Okazaki, R., T. Okazaki, and K. Sakabe. 1966. An extracellular nuclease of *Bacillus subtilis*: some novel properties as a DNA exonuclease. Biochem. Biophys. Res. Commun. 22: 611.

84. Pastan, I., and R. L. Perlman. 1968. The role of the lac promotor locus in the regulation of β-galactosidase synthesis by cyclic 3',5'-adenosine monophosphate. Proc. Natl. Acad. Sci. U.S. 61: 1336.

85. Pasteur, L. 1881. De l'attenuation des virus et de leur retour a la virulence. C. R. Acad. Sci. (Paris) 92: 429.

86. Perlman, R. L., B. DeCrombrugghe, and I. Pastan. 1969. Cyclic AMP regulates catabolite and transient repression in *E. coli*. Nature 223: 810.

87. Perlman, R. L., and I. Pastan. 1968. Cyclic 3',5'-AMP: Stimulation of β-galactosidase and tryptophanase induction in *E. coli*. Biochem. Biophys. Res. Commun. 30: 656.

88. Perlman, R. L., and I. Pastan. 1968. Regulation of β-galactosidase synthesis in *Escherichia coli* by cyclic adenosine 3',5'-monophosphate. J. Biol. Chem. 243: 5420.

89. Pollock, M. R. 1962. Exoenzymes, p. 121. *In* I. C. Gunsalus and R. Y. Stanier (eds.), The Bacteria IV. Academic Press, Inc., N.Y.

90. Rall, T. W., and E. W. Sutherland. 1961. The regulatory role of adenosine-3',5'-phosphate. Cold Spring Harbor Symposia Quant. Biol. 26: 347.

91. Reich, E., R. M. Franklin, A. J. Shatkin, and E. L. Tatum. 1961. Effect of actinomycin D on cellular nucleic acid synthesis and virus production. Science 134: 556.

92. Richmond, M. H. 1959. Formation of a lytic enzyme by a strain of *Bacillus subtilis*. Biochim. Biophys. Acta 33: 78.

93. Richmond, M. H. 1959. Properties of a lytic enzyme produced by a strain of *Bacillus subtilis*. Biochim. Biophys. Acta 33: 92.

94. Robinow, C. F. 1960. Morphology of bacterial spores, their development and germination, p. 207. *In* I. C. Gunsalus and R. Y. Stanier (eds.), The Bacteria I. Academic Press, Inc., N.Y.

95. Rolfe, F., and M. Meselson. 1959. The relative homogeneity of microbial DNA. Proc. Natl. Acad. Sci. U.S. 45: 1039.

96. Roux, E. 1890. Bacteridie charbonneuse asporogene. Ann. Inst. Pasteur 4: 25.

97. Ryter, A. 1965. Etude morphologique de la sporulation de *Bacillus subtilis*. Ann. Inst. Pasteur 108: 40.

98. Ryter, A., and F. Jacob. 1964. Etude au microscope electronique de la liaison entre noyau et mesosome chez *Bacillus subtilis*. Ann. Inst. Pasteur 107: 384.

99. Schaeffer, P. 1961. Etude genetique, biochimique et cytologique de la sporulation de *Bacillus subtilis*. D.Sc. Thesis. Faculty of Science, University of Paris, Paris.

100. Schaeffer, P. 1969. Sporulation and the production of antibiotics, exoenzymes, and exotoxins. Bacteriol. Rev. 33: 48.

101. Schaeffer, P., and H. Ionesco. 1959. Sur la transformation de *Bacillus subtilis*. Compt. Rend. 249: 481.

102. Schaeffer, P., and H. Ionesco. 1960. Contribution a l'etude genetique de la sporogenese. Compt. Rend. 251: 3125.

103. Schaeffer, P., H. Ionesco, A. Ryter, and G. Balassa. 1963. La Sporulation de *Bacillus subtilis*: etude genetique et physiologique. Colloq. Intern. Centre Natl. Rech. Sci. (Paris) 124: 553.

104. Schildkraut, C. L., J. Marmur, and P. Doty. 1962. Determination of the base composition of deoxyribonucleic acid from its buoyant density in CsCl. J. Mol. Biol. 4: 430.

105. Schwartz, V., L. Goldberg, G. M. Komrower, and A. Holzel. 1956. Some disturbances of erythrocyte metabolism in galactosaemia. Biochem. J. 62: 34.

106. Siminovitch, L., and A. F. Graham. 1955. Synthesis of nucleic acid in *Escherichia coli*. Can. J. Microbiol. 1: 721.

107. Skalka, A. 1969. Comparisons on the distribution of nucleotides in various phage DNA's, p. 55. *In* Abstracts of Papers Presented at the 1969 Bacteriophage Meetings, Cold Spring Harbor Laboratory of Quantitative Biology, Cold Spring Harbor, N.Y.

108. Skalka, A., E. Burgi, and A. D. Hershey. 1968. Segmental distribution of nucleotides in the DNA of bacteriophage lambda. J. Mol. Biol. 34: 1.

109. Smith, I. 1969. Chromatographic and electrophoretic techniques, 3rd ed. N.Y. Interscience Publisher, N.Y.

110. Smith, E. E. B., and G. T. Mills. 1954. Uridine nucleotide compounds of liver. Biochim. Biophys. Acta 13: 386.

111. Spizizen, J. 1958. Transformation of biochemically deficient strains of *Bacillus subtilis* by deoxyribonucleate. Proc. Natl. Acad. Sci. U.S. 44: 1072.

112. Spizizen, J. 1965. Analysis of asporogenic mutants in *Bacillus subtilis* by genetic transformation, p. 125. *In* L. L. Campbell and H. O. Halvorson (eds.), Spores III. American Society for Microbiol., Ann Arbor, Michigan.

113. Spotts, C. R., and J. Szulmajster. 1962. Synthese de l'acide ribonucleique et des proteines chez *Bacillus subtilis* sporogene et asporogene. Biochim. Biophys. Acta 61: 635.

114. Stanley, J. L., and H. Smith. 1961. Purification of factor I, and recognition of a third factor of the anthrax toxin. J. Gen. Microbiol. 26: 49.

115. Strange, R. E., and F. A. Dark. 1957. Cell-wall lytic enzymes at sporulation and spore germination in *Bacillus* species. J. Gen. Microbiol. 17: 525.

116. Sueoka, N., J. Marmur, and P. Doty. 1959. Heterogeneity in deoxyribonucleic acids. II. Dependence of the density of deoxyribonucleic acids on guanine plus cytosine content. Nature 183: 1429.

117. Sutherland, E. W., and T. W. Rall. 1960. The relation of adenosine-3',5'-phosphate and phosphorylase to the actions of catecholamines and other hormones. Pharmacol. Revs. 12: 265.

118. Szulmajster, J., R. E. Canfield, and J. Blicharska. 1963. The action of actinomycin D on the sporulation of *Bacillus subtilis*. Compt. Rend. 256: 2057.

119. Szulmajster, J., and R. S. Hanson. 1965. Physiological control of sporulation in *Bacillus subtilis*, p. 162. *In* L. L. Campbell and H. O. Halvorson (eds.), Spores III. American Society for Microbiol., Ann Arbor, Mich.

120. Takahashi, I. 1965. Transduction of sporogenesis in *Bacillus subtilis*. J. Bacteriol. 89: 294.

121. Takahashi, I. 1965. Localization of spore markers on the chromosome of *Bacillus subtilis*. J. Bacteriol. 89: 1065.

122. Takahashi, I. 1965. Mapping of spore markers on the chromosome of *Bacillus subtilis*, p. 138. *In* L. L. Campbell and H. O. Halvorson (eds.), Spores III. American Society for Microbiol., Ann Arbor, Mich.

123. Takahashi, I. 1966. Joint transfer of genetic markers in *Bacillus subtilis*. J. Bacteriol. 91: 101.

124. Takahashi, I. 1969. Genes controlling sporulation in *Bacillus subtilis*, p. 102. *In* L. L. Campbell (ed.), Spores IV. American Society for Microbiol., Bethesda, Maryland .

125. Trucco, R. E., R. Caputto, L. F. Leloir, and N. Mittelman. 1948. Galactokinase. Arch. Biochem. 18: 137.

126. Waites, W. M., D. Kay, I. W. Dawes, D. A. Wood, S. C. Warren, and J. Mandelstam. 1970. Sporulation in *Bacillus subtilis*. Correlation of biochemical events with morphological changes in asporogenous mutants. Biochem. J. 118: 667.

127. Wang, J. C., V. S. Nandi, D. S. Hogness, and N. Davidson. 1965. Isolation of λ dg deoxyribonucleic acid halves by Hg(II) binding and Cs_2SO_4 density-gradient centrifugation. Biochem. 4: 1697.

128. Warnaar, S. O., and J. A. Cohen. 1966. A quantitative assay for DNA-DNA hybrids using membrane filters. Biochem. Biophys. Res. Commun. 24: 554.

129. Weigle, J. J. 1960. Mutations affecting the density of bacteriophage λ. Nature 187: 161.

130. Westmoreland, B. C., W. Szybalski, and H. Ris. 1969. Mapping of deletions and substitutions in heteroduplex DNA molecules of bacteriophage lambda by electron microscopy. Science 163: 1343.

131. Wilkinson, J. F. 1949. The pathway of the adaptive fermentation of galactose by yeast. Biochem. J. 44: 460.

132. Willis, A. T. 1964. Anaerobic bacteriology in clinical medicine. Butherworths Publishing Co., London, England.

133. Wise, J., A. Swanson, and H. O. Halvorson. 1967. Dipicolinic acid-less mutants of *Bacillus cereus*. J. Bacteriol. 94: 2075.

134. Wu, R., and A. D. Kaiser. 1967. Mapping the 5'-terminal nucleotides of the DNA of bacteriophage λ and related phages. Proc. Natl. Acad. Sci. U.S. 57: 170.

135. Yamagishi, H. 1970. Nucleotide distribution in the DNA of *Escherichia coli*. J. Mol. Biol. 49: 603.

136. Yamagishi, H., and I. Takahashi. 1968. Genetic transcription in asporogenous mutants of *Bacillus subtilis*. Biochim. Biophys. Acta 155: 150.

137. Young, F. E. 1966. Fractionation and partial characterization of the products of autolysis of cell walls of *B. subtilis*. J. Bacteriol. 92: 839.

138. Young, F. E., and A. P. Jackson. 1966. Extent and significance of contamination of DNA by teichoic acid in *Bacillus subtilis*. Biochem. Biophys. Res. Commun. 23: 490.

139. Young, I.E., and P. C. Fitz-James. 1959. Chemical and morphological studies of bacterial spore formation. I. The formation of spores in *Bacillus cereus*. J. Biophysic. Biochem. Cytol. 6: 467.

140. Young, I. E., and P. C. Fitz-James. 1959. Chemical and morphological studies of bacterial spore formation. II. Spore and parasporal protein formation in *Bacillus cereus* var. Alesti. J. Biophysic. Biochem. Cytol. 6: 483.