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The nucleotide constitution of penicillin-induced protoplasts of Escherichia Coli

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INTRODUCTION

The actions of penicillin on bacteria and the correlation between the changes in morphology and viability produced and underlying metabolic alterations have been the subject of intensive investigations directed toward discovering the "mechanism of action" of the antibiotic. This phrase is usually intended to refer to the primary interaction of penicillin in the metabolic processes of the bacterial cell. The difficulty of separating this initial interaction from the extensive secondary sequence of biochemical alterations resulting both from the primary action and from the subsequent death of the bacterium has led to studies of the effect of the drug on diverse and seemingly distantly related phases of bacterial metabolism and given rise to much disagreement and confusion (12, 22, 92, 98). Recent investigations, however, have suggested that the alterations in nucleotide metabolism and in the structure and function of the cell wall seen in bacteria treated with penicillin may have a common biochemical basis and that the many other changes which occur may be consequences of these primary alterations.[1]

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[1]. The investigations discussed in this section are only a fraction of the many studies of the effects of penicillin on bacteria. A number of reviews are available (12,22,92,98) and the work cited is intended chiefly to serve as background in evaluation of the experiment reported. Since literature in much of this field is voluminous,references are given either to individual investigations which are frequently typical of those of several workers or, where possible, to review articles.

The action of penicillin on bacterial nucleic acid and nucleotide metabolism has been the subject of many studies. Interest in this subject dates from the early discovery that the drug in minimally bacteriostatic concentrations greatly decreased ability to metabolize exogenous ribonucleic acid (RNA) (42) and ribonucleotides such as guanylic and uridylic acid (30). These effects (measured by oxygen consumption after substrate addition) occurred within the first hour after addition of the antibiotic and preceded inhibition of RNA and protein synthesis and loss of ability to catabolize glucose or other sugars (31). In keeping with the well known greater bactericidal action of penicillin under conditions permitting cell division proliferating bacteria were found to be much more strongly affected than resting cultures.

Concurrently it was observed that in Staphylococcus aureus bacteriostatic doses of penicillin produced an almost immediate accumulation of low molecular weight compounds containing acid-labile phosphate (61) and a simultaneous rapid increase in the quantity of soluble nucleotides within the cell (56). The nature of the accumulating substances was clarified by the isolation and characterization by Park (62) of three unique nucleotides from susceptible penicillin-inhibited staphylococci. Each contained uracil, ribose, an acid-labile and acid-stable

phosphate group, and a then unknown amino sugar. In addition one of the compounds also contained one molecule of alanine and another alanine, lysine and D-glutamic acid in the ratio 3:1:1. Park proposed that these nucleotides were composed of uridine diphosphate (UDP) linked to the sugar and peptide. Almost simultaneously Strominger (84) noted that penicillin caused an increase in acetylhexosamine in the acid-soluble fraction of Lactobacillus helveticus, and others showed that E. coli treated with penicillin released soluble ultraviolet absorbing material into the medium on lysis (7,33).

While this work was in progress, other investigators studied the binding of penicillin by bacteria and the effects of the drug on the structural and osmotic functions of the cell wall. Penicillin was found to be specifically and irreversibly concentrated and bound by many organisms (50,69) and inhibition of cell division could be approximately correlated with the number of molecules bound per cell (18,21). Thus to achieve a 99 per cent inhibition of growth of organisms such as the pneumococcus and Streptococcus fecalis required that cells of the two species bind the same number of penicillin molecules although the sensitivities of the two (and hence the antibiotic concentration in the medium required to achieve this binding) were widely different. In keeping with the selective bacterial toxicity of penicillin and tending to emphasize the importance of this phenomenon,

neither mammalian cells in tissue culture (20) nor yeasts (11, 50) bound appreciable quantities of the drug. (Binding, however, is not the sole determinent of sensitivity since laboratory produced resistant mutants, in contradistinction to the naturally occurring resistant strains, continued to bind the drug as did the original sensitive strain from which they were derived (19)). Following these observations many attempts were made to isolate the penicillin binding site within the bacterial cell. Although complete success was not achieved, the finding by Cooper and others (10, 11, 23) that binding was associated with a lipid fraction probably situated near the cell wall suggested the bactericidal action of the drug resulting from an effect on this substance.

Studies of the action of penicillin on ability of bacterial cells to concentrate various metabolites and maintain their selective permiability lent additional support to the belief that the antibiotic acted on the cell wall. Gale (25) demonstrated that it rapidly halted the concentration of glutamic acid by sensitive staphylococci while protein synthesis from glutamic acid already within the bacterium continued unchanged. Penicillin resistance in this organism was shown to be associated with ability to synthesize glutamic acid and consequent independence of need to concentrate this amino acid from exogenous sources (26). Soon afterwards, others demonstrated

that penicillin either decreased ability to concentrate or increased permiability to a large number of diverse substances (98). (Gale had suggested that interference with ability to concentrate glutamic acid was the primary metabolic action of penicillin. Although Hotchkiss (35) showed that a different sensitive strain under slightly different conditions continued to concentrate this and other amino acids even though growth had been inhibited, the effect of the antibiotic on concentrating ability demonstrated by Gale and others is nevertheless quite definite.) Following these studies others investigated the alterations in bacterial electrostatic charge caused by penicillin in an attempt to learn more about its effect on bacterial surface properties. Although the mechanism or importance of the phenomenon is not clear, it was interesting that even sublethal amounts of the drug did greatly alter the electrophoretic mobility of staphlyococci (52).

Almost immediately after the modern rediscovery of penicillin a great many workers began to observe the changes it produced in bacterial morphology. It was soon found that coliform bacilli treated with the antibiotic tended to elongate and developed rounded swellings (28, 29). Detailed observation of these changes showed that in species such as E. coli, S. typhosa and A. aerogenes, penicillin produced first elongation and then fusiform swellings leading to

complete rounding and continued enlargement and finally to vacuolization and eventual death by lysis (17). (The changes in gram positive cocci were perhaps similar but harder to observe because of the original round shape.) These alterations were interpreted by the observer (17) as indicating that penicillin "interferes specifically with the formation of the outer supporting cell wall while otherwise allowing growth to proceed until the organism finally bursts its defective envelope and so undergoes lysis". Electron microscopy of the changes in coliform bacteria showed changes in the cell wall prior to lysis and tended to support this early conclusion (36). Other workers demonstrated that when S. typhosa (14), Proteus vulgarus (13), or other gram negative rod organisms (15) were treated with properly adjusted sublethal amounts of penicillin in suitably enriched agar, the rounded swollen forms produced would develop into small irregular protoplasmic masses (named L-forms) which could be propagated as such. Upon removal of penicillin they would revert first to the large round forms and then to the normal rod form and divide. These L-forms can be produced by other substances (although penicillin is perhaps the best agent), and their origin and significance is not clear. However, it would seem that in inducing their formation, penicillin is definitely interfering with cell wall production and/or function while still permitting the remainder of the bacterium to divide

and reproduce. Electron microscopic studies of the L-forms (66) tended to support this conclusion.

Two recent contributions have suggested that these alterations in nucleotide metabolism, cell wall function, surface properties, and morphology are all the result of a single biochemical action of the drug. The first of these was the discovery by Lederberg (44, 45) and others (32) that the penicillin-produced osmotically fragile round forms of E. coli (47) can be stabilized by the addition of hypertonic sucrose to the medium to prevent lysis. When a fully grown culture of E. coli is diluted into an enriched growth medium containing 100 to 1,000 units of penicillin per milliliter and 20 per cent sucrose, morphologic changes almost identical to those earlier described occur, but extensive swelling or lysis do not take place. The resulting three to six micron spheres are lysed by distilled water but can be preserved in the concentrated sucrose or in other hypertonic medium. Upon removal from penicillin medium, they will revert to the rod form and divide. If these round forms are plated in suitably prepared solid sucrose agar, L-forms similar to those observed earlier in other coliform species can be produced (46).

Since it was believed that these stabilized round forms lacked all or a portion of their cell wall, they were referred to as "protoplasts" (44). This term is derived from a botanical designation for the protoplasm

contained within the cell wall in higher plants. It was first used in bacteriology to refer to the lysozyme-produced round forms which have many properties similar to those produced by penicillin. The structural and biochemical relationship of these two types of protoplasts is not clear, and use of the term for the penicillin forms has been questioned(43,94,73). Since the fundamental alterations in cell wall structure induced by these agents have not been elucidated and since there is evidence that lysozyme probably alters the same cell wall component affected by penicillin (72), the term will be used throughout in this discussion. This does not imply a necessary identity of these variously produced protoplasts; the term is simply taken as a convenient designation for a spherical bacterial cell with probable alterations in cell wall structure or function and properties similar to those described above. Unless stated otherwise, however, the word 'protoplast' for simplicity is used to refer to the penicillin protoplasts of Lederberg. With increased knowledge of underlying alterations in the protoplasts produced by different substances, a revision in terminology may be desirable.

After the isolation of the hexosamine—peptide nucleotides from sensitive penicillin inhibited staphlyococci, a number of components of these nucleotides were identified as elements of bacterial cell walls. Harris and

Cummins (34) demonstrated the presence of glucosamine and an acetylamino sugar originally detected in spores of certain bacteria (82) in the cell walls of many gram positive bacteria. Later, structural determinations (83) suggested that this acetylamino sugar was probably 3-O-carboxyethyl N-acetylglucosamine. The deacetylated compound has been named muramic acid, and its structure has been confirmed by synthesis (41). Other workers showed that many bacterial cell walls contained large quantities of D-glutamic acid, lysine, α,ϵ-diamino-pimelic acid (DAP), and glycine (71, 53, 97).

The probable relationship of the hexosamine nucleotides to cell wall synthesis was elucidated by the discoveries of Park and Strominger (64) that the sugar of the nucleotide was identical with N-acetylmuramic acid from the cell wall and that it and the amino acids of the nucleotide were present in the same ratio in the nucleotide and in the cell wall of the staphylococcal strain from which it was derived. They proposed the formula given in figure 1 as representing the probable structure of the full pentapeptide nucleotide. In the light of their work and that of Lederberg, they suggested that the primary action of penicillin was to block a step in synthesis of the bacterial cell wall and hence prevent transfer of the acetylmuramic acid peptide from the nucleotide to the wall.

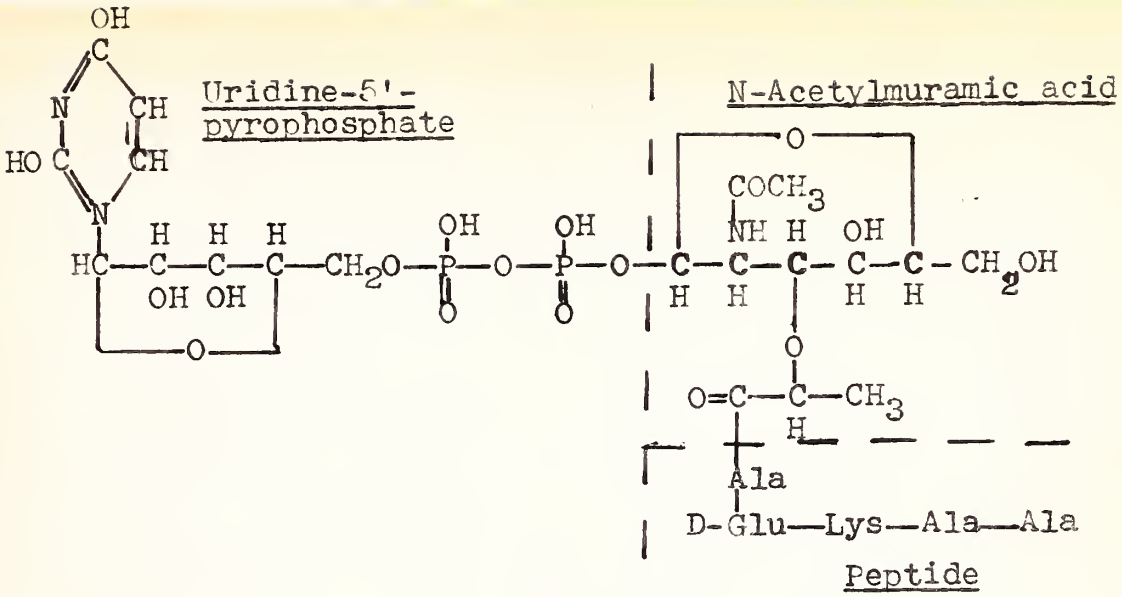

Figure 1. The pentapeptide nucleotide that accumulates in penicillin-treated sensitive Staphylococcus aureus (62, 63).

The defect in the wall so produced was presumed to lead to loss of the protective barrier between the high intracellular osmotic pressure and the medium and (unless prevented by the concentrated sucrose solutions) to death of the bacterium by osmotic lysis. The observations that nucleotide accumulation is much greater in actively proliferating cultures (85) and that the protoplasts are formed only in such cultures (44) are in keeping with the well-known greater sensitivity to penicillin of dividing bacteria.

* * *

In the light of this formulation, it was decided to study the nucleotides of the penicillin protoplasts and perhaps thereby learn more both of the action of the drug on nucleotide metabolism and of the biochemical role of

the amino acid-hexosamine nucleotides. Because the protoplasts are cells with a clear structural (and probably metabolic) lesion yet are able to synthesize enzymes (75, 80) and are otherwise seemingly minimally altered, they would appear to be well suited for such a study. Besides their occurrence in penicillin inhibited staphylococci, these uridine nucleotides have been detected more tentatively in small amounts in normal staphylococci (39, 85), normal and penicillin, inhibited lactobacilli (4, 85), and streptococcus hemolyticus (51), and the diaminopimelic acid requiring mutant of E. coli (89). Similar compounds containing glycine also probably appear in the staphylococcus in the presence of penicillin (39). Accordingly it would be of considerable interest to know whether they appear in a wild type gram negative resistant organism either normally or when the cell sustains a penicillin induced lesion in wall polymer synthesis. In view of earlier work demonstrating other abnormalities of nucleotide metabolism brought about by the antibiotics, it seemed worthwhile to study in addition the other nucleotides of the protoplasts.

The work described, therefore, is a comparison of the total amount and relative composition of the nucleotides of the acid-soluble fraction of normal E. coli and the penicillin protoplasts produced from the same strain. The amounts of ribonucleic acid, desoxyribonucleic acid (DNA)

and protein in cultures were measured primarily to serve as a base for quantitative comparison of the nucleotides and also to detect any major change that might occur in the relative proportion of these constituents. Emphasis was placed on measuring the relative total quantities of acid-soluble nucleotides in cultures, discovering any major qualitative or quantitative difference between the nucleotides of the normal cells and protoplasts, and determining the possible presence, relative amount, and composition of any amino acid or hexosamine containing nucleotide.

MATERIALS AND METHODS

A. Culture of Organism: Escherichia coli (A.T.C.C. 9637) was maintained in stock cultures on agar slants and used in all experiments described.

Two culture media were employed. Medium E contained inorganic salts and glucose and was prepared by dilution of stock buffer as described by Vogel (93). Medium A (designed to be similar to Difco Penassay Medium used by Lederberg (44)) had the following composition:

(a)	Difco Bacto Yeast Extract	1.5 g.
	Difco Bacto Nutrient Broth	6.5 g.
	$MgSO_4.7H_2O$	4.2 g.
(b)	Medium E Stock Buffer (93)	20.0 ml.
(c)	Sucrose (commercial granulated sugar)	200 g.
(d)	Glucose	5.0 g.
	Distilled Water to make	1,000 ml.

If the first two groups are autoclaved together in the distilled water, a light yellow precipitate usually forms. Although this precipitate will always redisolve to give a clear medium with apparently unaltered properties, groups (a), (b), and (c) were usually autoclaved separately in appropriate volumes of water. Glucose was always added after autoclaving as a sterile 50 per cent solution.

Inoculation cultures for all experiments were small (5 to 20 ml.) late-logarithmic phase cultures grown in medium E from agar slants. To prepare intact organisms for nucleotide fractionation, large volumes of medium A were

were inoculated and incubated at room temperature. (For this purpose only medium A was diluted with one third volume of medium E to give a culture medium identical with that used for protoplast preparation). Growth was followed by increase in optical density at 660 mμ and the bacteria were harvested in late logarithmic phase (usually after six to nine hours). In all cases the mixtures were incubated either with shaking in Erlenmeyer or three-liter tapered bacterial culture flasks or with vigorous aeration in eight-liter bottles to which a small amount of silicone gel had been added to prevent excessive foaming.

B. Preparation of Protoplasts: For this purpose bacteria were grown overnight in medium E at 37°. One volume of such a culture was added to three volumes of medium A supplemented with 1,000 units per milliliter of crystalline potassium or sodium penicillin G. The mixture was then incubated at room temperature either in a culture flask with shaking (for amounts up to two liters) or in an eight-liter bottle with vigorous aeration. (Silicone gel was added when necessary to diminish foaming). Under such conditions the changes in morphology and optical density were almost identical to those described and pictured by Lederberg (44,46). Incubation was continued until gram- or methylene blue-stained slides showed 80 to 90 per cent rounded or slightly irregular three to five micron spheres with no residual rod-like protrusion (five to eight hours depending on the volume of the incubation

mixture).

C. Harvesting and Separation of Cell Components: The intact bacteria or protoplasts were harvested in either a Sharples or conventional centrifuge and washed three times with a solution of 20 per cent sucrose and 0.2 per cent $MgSO_4$. In all cases gram or methylene blue stains were made immediately after washing and showed no significant change from those made prior to harvesting. The acid-soluble fraction was extracted by mixing the sedimented cells at 0° with two to three volumes of perchloric acid (PCA). The amount added was calculated to give a resulting concentration of approximately 6 per cent, and the mixture was stirred gently for approximately fifteen minutes.

The acid-soluble fraction was then separated by centrifugation at 4° and neutralized to pH 7 with potassium hydroxide. After standing at least one hour at 0° C the usually light green color solution was removed from the insoluble potassium perchlorate. The perchloric acid insoluble portion of the cells was washed three times with approximately four volumes of cold PCA and heated thirty to forty-five minutes in a boiling water bath with a similar volume of 6 per cent perchloric acid. After separation from the hydrolyzed nucleic acids by centrifugation, the protein precipitate was disolved in 0.5-1.0 N NaOH. Usually gentle warming for thirty minutes to one hour was required.

D. Determination of RNA, DNA, and Protein: Dilutions of

the above solutions were made and suitable aliquots taken. RNA was determined by the orcinol method (74), DNA by the diphenylamine reaction (16), and protein by the Folin reaction (49). After dilution the usual brown color of the dissolved protein and nucleic acid was found to have a negligibly small optical density at the wave lengths used in these methods. Adenylic acid (AMP) and a preparation of purified calf thymus DNA were used as standards for the nucleic acid determinations. Orcinol determinations done on the last PCA washing prior to hydrolysis of the nucleic acids showed negligible interference by residual sucrose. For the protein measurements the standard employed was crystalline bovine albumin either prepared as a dilute solution immediately before use or freshly diluted from a previously prepared concentrated solution. After dilution of the alkaline protein solution from the cells, the hydroxyl ion concentration was always less than 0.01 N and the alkaline copper reagent (reagent C) was therefore used in the determinations.

E. Fractionation of Acid-soluble Nucleotides: The procedure of Hurlbert (37) of gradient elution with formic acid and ammonium formate from Dowex-1 (formate) resin was used throughout. The volume and optical density at 260 and 280 mμ of the nucleotide solution was measured prior to absorption on the column. Sufficient resin was used to give a column with one to two centimeter diameter and

five to fifteen centimeter length depending on the total absorption of the nucleotide solution. One mixer of either 400 or 800 ml. was used, and the column was eluted with approximately two mixer volumes of 4 N formic acid and then successively with equal volumes of 0.2, 0.4, and 0.8 N ammonium formate in 4 N formic acid. (Frequently the 0.2 N ammonium formate solution was omitted unless a particularly fine fractionation was desired). Five to fifteen mililiter fractions were collected and the optical density of all fractions significant ultraviolet absorption was read at 260 and 280 mμ and ratios were computed. The fractions comprising the various absorption peaks were pooled; and, after their volume and optical density at 260 mμ had been recorded, were evaporated to dryness under reduced pressure. (When ammonium formate was present in the pooled fractions, they were first passed over activated charcoal colums. The colums were washed with distilled water and the nucleotides then eluted with 50 to 100 ml. of a mixture of ethanol and concentrated ammonium hydroxide (7:3), and then evaporated to dryness.)

F. Enzymatic Assay of Diphosphopyridine nucleotide (DPN): The fraction directly from the column was neutralized to pH 7 and suitable volumes were assayed using a modification of the alcohol dehydrogenase (ADH) method of Racker (9,68). In a Beckman spectrophotometer cuvette were mixed 0.3 mL, 1 M tris buffer pH 8; 0.01 of a yeast ADH preparation; the

solution to be assayed, and distilled water to make 3.0 ml. A standard containing 0.2 ml. of a standard solution of DPN and a blank containing 0.2 ml. of distilled water in place of the unknown solution were also prepared. After addition of 0.01 ml of 95 per cent ethanol to the mixtures, the optical density at 340 mμ was followed. After maximum increase had occurred (usually 30 minutes) the DPN content of the sample was calculated from the comparative increase of the standard and unknown.

G. Hydrolysis and Paper Chromatography of Nucleotides and their Components: The solvent systems employed for chromatography of unhydrolyzed nucleotides were butanol-acetic acid-water (2:1:1) and a propanol-ammonium sulfate solvent (60). Evaporated fractions from the ion exchange column were partially purified by applying the material as a band across Whatman number two paper, running in butanol-acetic acid, and cutting out the ultraviolet absorbing strips and eluting with water. All chromatograms were run descending in cylindrical glass tanks, and for all other purposes Whatman number one paper was used.

After hydrolysis of nucleotides for eight hours in 6 N HCl in a sealed tube at 100°, residual acid was removed by evaporation to dryness several times. The following solvent systems were utilized for detection of components of the hydrolysates: butanol-acetic acid-water (2:1:1 and 4:1:5) (65), phenol-water (4:1 with small volume dilute

NH_4OH in tank), phenol-water (3:1) and 2, 4-lutidine (technical grade)-water (3:2) (62), methanol-pyridine-10 N HCl-water (80-10-2.5-17.5), (71) and butanol-ethanol-water (4:1:1). All ratios given are by volume. Phenol was distilled within two weeks of using and stored at 4°C. In the resultant chromatograms purines and pyrimidines were detected by their ultraviolet absorption, and amino acids and hexosamines by dipping the papers in or spraying with 0.5 per cent ninydrin in ethanol or a similar solution containing collidine (48). Hexosamines were also detected by a modification (79) of the Elson-Morgan method of Partridge (65). In using this procedure it was found that the pink spots were best seen immediately after the second (dimethylaminobenzaldhyde) dipping and invariably faded and became more obscure after the second heating. The method gave quite good results in that 5 to 10 micrograms of hexosamine yielded readily discernable color whereas 20 micrograms of alanine gave only faint or delayed colors and did not interfere.

After hydrolysis of the nucleotides for three hours in 1 N HCl, attempts were made to detect hexosamines by the pyridine-ninhydrin decarboxylation procedure (81). This was invariably unsatisfactory as, although purple colors were often formed in the pyridine heating step, (indicating the presence of hexosamines) the later chromatograms were usually obscured by diffuse white spots (probably of salt) which did

not darken after the silver nitrate dipping step.

The standards used in chromatography were commercial nucleotides, purines and pyrimidines, D-glucosamine, single amino acids, and the amino acid mixtures of Levy and Chung (48). The latter separated well in the 2:1:1 butanol-acetic acid system and gave useful though incomplete separations in the phenol-water and lutidine-water systems. Since no muramic acid was available as a reference compound the mobilities of the hexosamines detected were measured relative to alanine and glycine amino acid standards and compared with values given by others for these relative mobilities in identical solvent systems (2,4,62).

RESULTS

A. Protein and Nucleic Acid of Protoplasts and Intact Cells. The quantities of protein, RNA, and DNA in each of the protoplast and normal cell cultures are given in table I. For comparison of results in the two forms, the relative amounts of nucleic acid per milligram of protein have been calculated. It may be seen that the ratio of RNA to protein is the same in the two forms and that the DNA determinations do not have sufficient precision to allow meaningful comparison. (The maximum differences which could have occurred undetected with the error observed in the determinations is given in the bottom line of the table). The large variation in the DNA measurements is probably a consequence of the smaller amount of this substance present and the therefore relatively larger interference by other substances (perhaps sucrose not removed by washing) in the hydrolyzed nucleic acid solutions. Sucrose was found to react with diphenylamine, and unfortunately no determinations were done on the last perchloric acid washing to estimate the possible source of interference.

B. Characterization of Nucleotides in the Protoplasts and Normal Bacteria. The patterns of nucleotide elutions in the two forms may be seen in the representative diagrams of figures 2 to 4 showing the ultraviolet absorption of successive fractions from the ion exchange column. Early in the elution both protoplasts and intact cells yielded first one small and then two extremely large fractions (the latter numbered

TABLE I

Protein and Nucleic Acid Content of Normal Cells and Protoplasts

Elution No.	Protein (mg. bovine albumin)	RNA (mg. AMP)	DNA (mg. DNA)	RNA / Protein	DNA / Protein
Protoplasts					
P-1-58	330	88.5	30.3	0.27	0.092
P-2a-58	670	108	38.6	0.16	0.058
P-2d-58	900	133	63.5	0.15	0.070
P-3a-58	650	171	138	0.26	0.210
P-3b-58	740	110	24.4	0.15	0.033
P-6-58	5,000	1,130	221	0.23	0.044
P-7-58	5,880	1,150	158	0.20	0.027
P-8-58	5,225	2,230	276	0.42	0.052
Mean				0.230	0.0537
±S.D.(of mean)				±0.046	±0.011
Normal Cells					
C-2-57	463	75.5	14.8	0.16	0.032
C-3-57	182	55.5	10.6	0.33	0.058
C-4-57	354	109.5	15.4	0.31	0.043
C-5-57	257	85.0	16.0	0.33	0.062
C-1-58	150	47.3	6.15	0.32	0.041
C-2-58	1,000	190	18.0	0.19	0.018
C-3-58	5,750	888	138	0.16	0.024
Mean				0.256	0.0397
±S.D.(of mean)				±0.046	±0.011
Difference (protoplast - normal)				not significant	
Minimum Detectable Difference*				0.098 (38%)	0.397 (61%)

*Minimum change that could have been detected (i.e. would have been statistically significant) with observed standard deviation of mean (p=0.05).

Figures 2-4

The acid-soluble material was extracted from a centrifuged 16-liter culture of E. coli and from penicillin protoplasts prepared from a similar sized culture of the same organism, and passed over a Dowex-1 (formate) resin column of approximately 1.5 cm. diameter and 8 cm. length. Shown in the diagrams is the optical density at 260 mμ of 10 to 15 ml. fractions successively eluted with a gradient of gradually increasing formate ion concentration. (Note change of absorption scale after the large initial peaks in figure 2.) Total absorption of the acid-soluble extract of the protoplasts was 1.6 times that of the intact bacteria. As discussed in the text, the major components of peaks 1 and 2 were DPN and AMP while peaks 3-5 yielded the amino acid-hexosamine fractions. The initial unnumbered peak preceding number 1 is probably cytidylic acid; those following peak 5 were not characterized. The change in eluting solution is marked with arrows: (a.) - 4N formic acid, (b.) - 0.2N ammonium formate in 4N formic acid, (c.) - 0.4N ammonium formate in 4N formic acid.

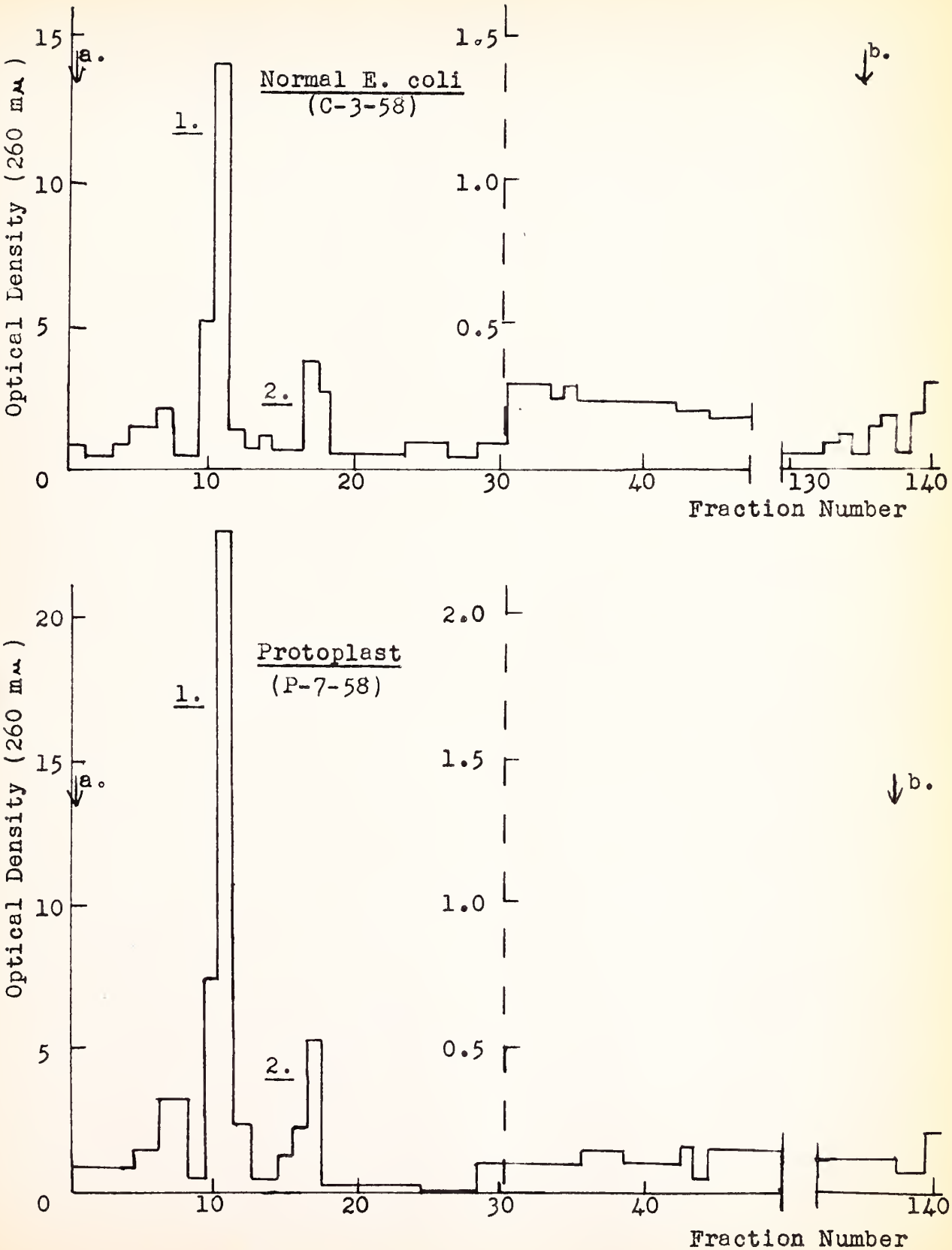

Figure 2. Elution diagram of normal E. coli and protoplast nucleotides. Section I.

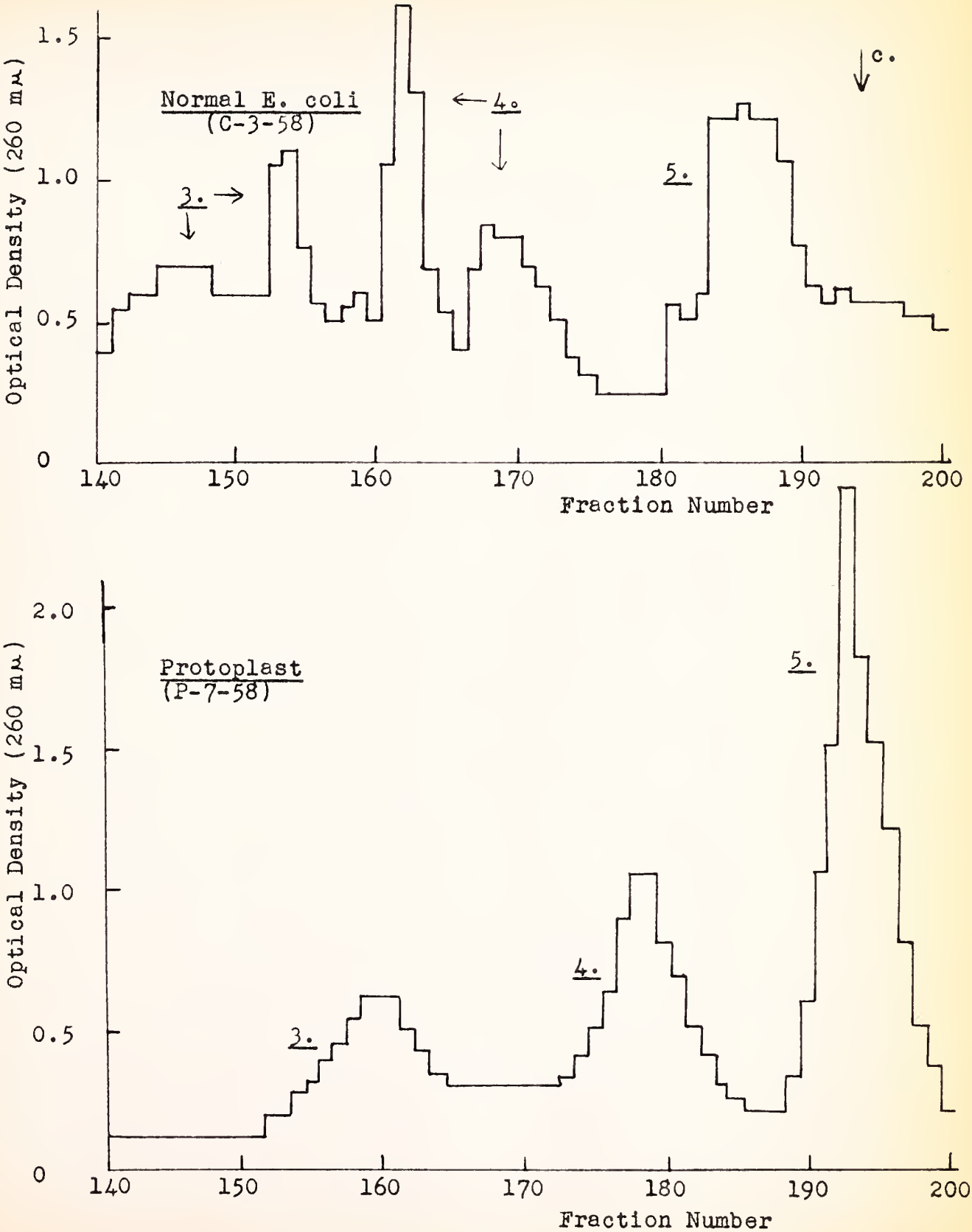

Figure 3. Elution diagram of normal E. coli and protoplast nucleotides. Section II.

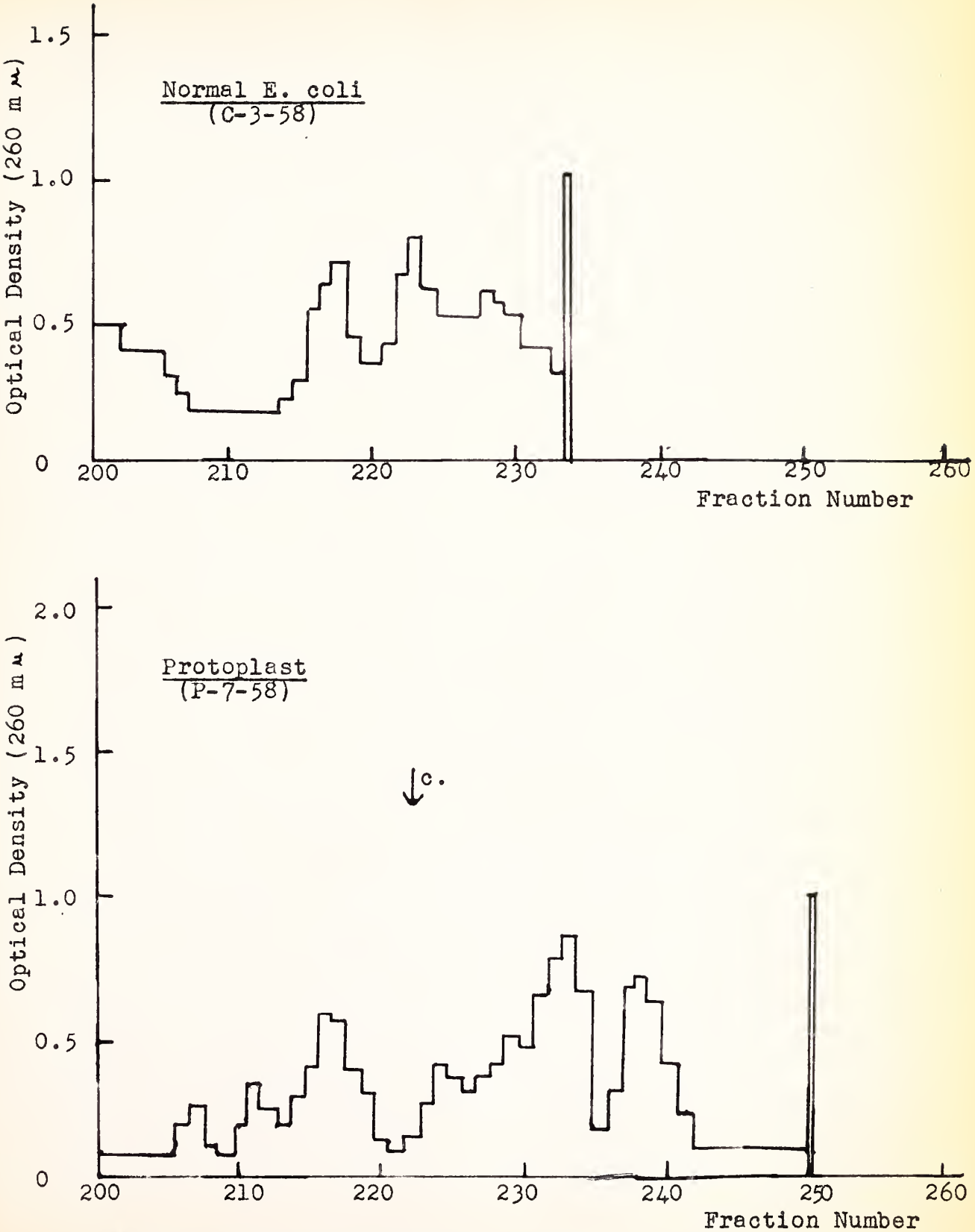

Figure 4. Elution diagram of normal E. coli and protoplast nucleotides. (Volume of fractions was doubled after arrow c.) Section III.

one and two in the diagrams). Their position and relative absorption at 280 and 260 mμ suggested that the principal component of fraction one was DPN and that of fraction two was adenylic acid. Chromatography in butanol-acetic acid and propanol-ammonium sulfare in each case demonstrated the presence of one principal ultraviolet-absorbing component with an Rf almost identical to standards of the substances mentioned. Usually these were the only ultraviolet absorbing materials present in the fractions; however, occasionally extremely faint spots were seen with the mobility of the principal component of the neighboring peak. To establish more definitively the composition of fraction one and determine the oxidation state of the DPN present, a small sample was purified by chromatography in butanol-acetic acid and assayed enzymatically. Comparison of its relative reduction by alcohol dehydrogenase and absorption at 260 mμ indicated that the material eluted from the paper was at least 96 per cent oxidized DPN.

The small initial (unnumbered) fraction was poorly separated from the large DPN peak. Its position and relatively high absorption at 280 mμ indicates that it is probably chiefly cytidylic acid, and chromatography in propanol-ammonium sulfate on one occasion yielded a faint spot with the mobility of this substance. Further identification was not attempted.

* * *

Following these large initial peaks, no more ultraviolet absorbing material was eluted by the 4 N formic acid. When ammonium formate was then used a series of much smaller peaks appeared which continued almost to the end of the elution. While the absorption patterns in this section (figures 3 and 4) are not identical, there was no major nucleotide component present in only one of the two cell forms. Of these smaller fractions, the only ones whose composition was investigated were those at the beginning of the ammonium formate portion of the elution. To obtain sufficient quantities for characterization, large numbers of protoplasts and normal bacteria had to be grown, and the results given are based on 12 to 16 liter original cultures. Usually three distinct fractions were found (three, four, and five in the diagrams). However, in one elution, in place of these three, one larger fraction appeared late in the 4 N formic acid section, which on subsequent chromatography yielded three subfractions probably identical to those usually obtained directly from the column. Analysis of these fractions was directed solely towards discovering the principal purine or pyrimidine component and identifying possible amino acids and/or hexosamines present. The procedures employed for purification prior to hydrolysis are given for each fraction in tables II and III. In most cases the material from the column was absorbed on and eluted from charcoal and frequently

further purified by chromatography in butanol-acetic acid. Except in the one instance noted, this chromatography separated the column fractions into a principal component of low mobility (Rf 0.08-0.15) and one or more faster moving substances. The latter had a total ultraviolet absorption after elution from paper less than 15 per cent of the principal component and were not further characterized.

After such purification, the nucleotides were hydrolyzed in 6 N HCl and the resulting ultraviolet absorbing and ninhydrin reacting substances identified by paper chromatography. From each elution either one or two fractions were obtained which yielded on hydrolysis one ultraviolet absorbing spot with an Rf identical to that of uracil and several distinct ninhydrin reacting substances. Two of the latter were identified as glycine and glutamic acid, and others seemed to have Rf's almost identical to those of alanine and serine. However, on spraying the papers with ninhydrin solution containing collidine the supposed alanine spot was seen to have a deep turquoise color distinctly different from that produced by an alanine standard. Since muramic acid also reacts with ninhydrin and since its Rf is almost identical with that of alanine in many systems (62), when sufficient material was available, chromatograms of the hydrolysates were treated with Elson-Morgan reagents. Comparison of the mobilities of the ninhydrin and Elson-Morgan reacting components showed that all fractions so

studied contained muramic acid, glucosamine, glycine, and glutamic acid. Because of the difficulty of detecting alanine in the presence of muramic acid, where sufficient material remained additional chromatograms were run in the lutidine-water system in which these substances are separable. Of the two protoplast and two normal cell nucleotides so studied, one (from normal bacteria) definitely contained alanine and a second (from protoplasts) gave a faint spot in the corresponding place. In the other fractions where insufficient material remained the presence or absence of this amino acid could not be definitively established.

The previously mentioned amino acids and amino sugars accounted for all the ninhydrin-positive substances in all of the protoplast and in one of the normal two cell fractions. However, the other normal cell hydrolysate on chromotography was seen to contain in addition a slower moving ninhydrin-reacting material with an Rf slightly less than that of lysine. When this material was eluted from the paper and rechromatographed in the methanol-pyridine-HCl system (designed for identification of diaminopimelic acid) it gave a definite green spot identical in color and Rf to a DAP standard. The identity of this substance was confirmed by the observation that in the lutidine-water system, the slow moving spot which it produced turned bright yellow on standing 24 hours. The spots of lysine and cysteine (which

have approximately the same mobility as DAP) remained a purple color whereas that of the diaminopimelic acid underwent a color change identical to that of the material from the bacterial fraction hydrolysate. In order to determine as definitely as possible the presence or absence of DAP either in the other normal cell nucleotide (from the same cell culture) or the four protoplasts nucleotides, these were chromatographed again either in the methanol-pyridine or lutidine-water systems. The normal cell fraction gave an only extremely faint spot corresponding to DAP while none of the protoplast chromatograms had any spot in this region.

A listing of the Rf's in various solvent systems and proposed identity of the components of the nucleotide fractions just discussed is given in tables II and III, and examples of the chromatograms obtained are shown in figures 5 to 7. Fractions containing amino acids and amino sugars were found in the one normal cell and three protoplast cultures which contained sufficient organisms to permit their characterization.[2] In summary, it may be stated that in both the protoplasts and intact cells the hydrolyzed

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[2]. In addition to these four large cultures, an earlier smaller normal cell elution (C-2-58) contained a fraction in the same position which on hydrolysis yielded uracil and four ninhydrin positive components. Although, because of the limited material available, it could not be further characterized, it is quite likely in retrospect, that it was similar to the fractions discussed above and it is therefore included as a normal cell amino acid nucleotide fraction in table four.

Tables II and III, Figures 5-7

Nucleotides were fractionated using the ion exchange resin as described in text. After partial purification by chromatography in butanol-acetic acid and elution from charcoal as in the tables, the various fractions were hydrolyzed in HCl and chromatographed. The resulting papers were inspected under ultraviolet light and treated with ninhydrin and Elson-Morgon reagents. Purines and pyrimidines cannot be seen in solvent systems which themselves have a high ultraviolet absorption; muramic acid and alanine cannot be detected in the presence of each other except in the lutidine system. The standards were always run in the same tank and almost always on the same paper as the unknowns.

The tables summarize the Rf and proposed identity of components of all fractions found to contain amino acids. The figures are representative chromatograms of the hydrolyzed fractions. It should be remembered that color of the spots which cannot be shown in these scale drawings was of great aid in identification of ninhydrin reacting substances.

Abbreviations are as follows: Ala--alanine, arg--arginine, asp--aspartic acid, cys--cysteine, DAP--α,ε-diaminopimelic acid, GA--glucosamine, glu--glutamic acid, gly--glycine, his--histidine, isoleu--isoleucine, leu--leucine, lys--lysine, met--methionine, mur--muramic acid, phe--phenylalanine, pro--proline, ser--serine, thr--threonine, tryp--tryptophan, tyr--tyrosine, val--valine.

Notes below refer to the tables.

* $R_{\text{alanine}} \times 100$ (i.e. mobility with respect to alanine standard.)

** $R_{\text{glycine}} \times 100$.

***No glucosamine standard was used on these chromatograms and Elson-Morgon determinations were not done. While these fractions probably are the same as those isolated later from other protoplast cultures, with the evidence attained, it is impossible to be certain of the presence or absence of alanine, muramic acid, or glucosamine.

(+)--component believed present.
(-)--component believed absent.

TABLE II

Fraction No. and Purification Procedure	Components of Normal Cell: Component	Present or Absent	Nucleotides Containing Amino Acids: Rfx100 in solvent systems given (Rfx100 of standards in parentheses) Butanol-Acetic Acid (2:1:1)	Phenol Water (NH_3)	Lutidine Water	Methanol Pyridine
C-2-58-F6 elution from charcoal and from butanol acetic acid paper	Uracil Unidentified ninhydrin reacting substances	+	56 (54)			
		+	43			
		+	36			
		+	30			
		+	23			
C-3-58-F4 elution from charcoal and from butanol-acetic acid paper	Uracil	+	53 (52)	----	----	
	Alanine	+	43 (43)	65 (65)	31 (30)	
	Glutamic acid	+	36 (36)	29 (27)	21 (21)	
	Glycine	+	30 (31)	48 (45)	25 (24)	
	Glucosamine (ninhydrin) (Elson-Morgon)	+	56 (56)*	100(100)*	171(164)*	
			54 (56)*	not done	170(164)*	
	Muramic Acid (ninhydrin) (Elson-Morgon)	+	----	no std.	171(170)*	
			95 (96)*	not done	170(170)*	
	DAP (whole fraction) (slowest two components eluted from butanol-acetic acid)	+	17 (not run)		12 (14) (yellow)	22 (22) green
C-3-58-F3,5 elution from charcoal and from butanol-acetic acid paper (combination of two fractions separated on column and mixed inadvertantly).	Uracil	+	53 (52)	----	----	
	Alanine	-	44	none(63)	none(32)	
	Glutamic Acid	+	35 (37)	30 (30)	18 (20)	
	Glycine	+	30 (33)	45 (45)	23 (22)	
	DAP (whole fraction) (slowest two components eluted from butanol-acetic acid)	-	none	none	none(14)	none (22) green
			no std.	no std.		
	Muramic Acid (ninhydrin) (Elson-Morgon)	+	96 (96)*	no std.	162(163)*	
			94 (96)*	not done	162(163)*	
	Glucosamine (ninhydrin) (Elson-Morgon)	+	52 (52)*	116(116)*	162(170)*	
			51 (53)*	no std.	162(170)*	

III

Components of Protoc... tides Containing Amino Acids

Fraction No. and Purification	Components	Presen Abse	Rfx100 in solvent systems given (Rfx100 of standards in parenthesis)			
			Butanol Acetic Acid (2:1:1)	Butanol Acetic Acid (4:1:5)	Lutidine Water	Methanol Pyridine
F-6-58-F4A elution from butanol-acetic acid-water paper	Uracil	+	53 (51)			--------
	Alanine	***	47 (45)*			78 (77)
	Glutamic Acid	+	43 (41)			60-70 (72)
	Glycine	+	34 (34)			60-70 (60)
	DAP					none (52)
	Glucosamine (ninhydrin)	***	23(none)			? no standard run
F-6-58F4B elution from butanol-acetic acid-water paper	Uracil	+	61 (60)			--------
	Alanine	***	46 (45)			79 (77)
	Glutamic Acid	+	42 (41)			60-70 (72)
	Glycine	+	34 (34)			60-70 (60)
	DAP	-				none (52)
	Glucosamine (ninhydrin)	***	23 (-)			? no standard run
P-7-58-F3 elution from charcoal column	Uracil	+	58 (59)	54 (53)		--------
	Alanine	trac	none(45)			32 (31)
	Glutamic Acid	+	46 (47)			20 (21)
	Glycine	+	37 (37)			25 (27)
	DAP	-				none(14)
	Glucosamine (ninhydrin) (Elson-Morgon)	+	55 (54)*			165(162)* 165(162)*
	Muramic Acid (ninhydrin) (Elson-Morgon)	+	102 (96)* 98 (96)*			165(170)* 165(170)*
P-8-58-F2 elution from charcoal column	Uracil	+	63 (59) (50)	46 (48) (28)		--------
	Alanine	-				none(30)
	Glutamic Acid	+	46 (47)	22 (23)		20 (19)
	Glycine	+	37 (37)	16 (16)		25 (24)
	DAP	-		none(4) yellow		none(14) yellow
	Glucosamine (ninhydrin) (Elson-Morgon)	+	54(none)* 52 (54)*	75 (75)** not done		172(170)* not done
	Muramic Acid (ninhydrin) (Elson-Morgon)	+	106 (96)* 98 (96)*	176(181)** not done		172(170)* 172(170)*

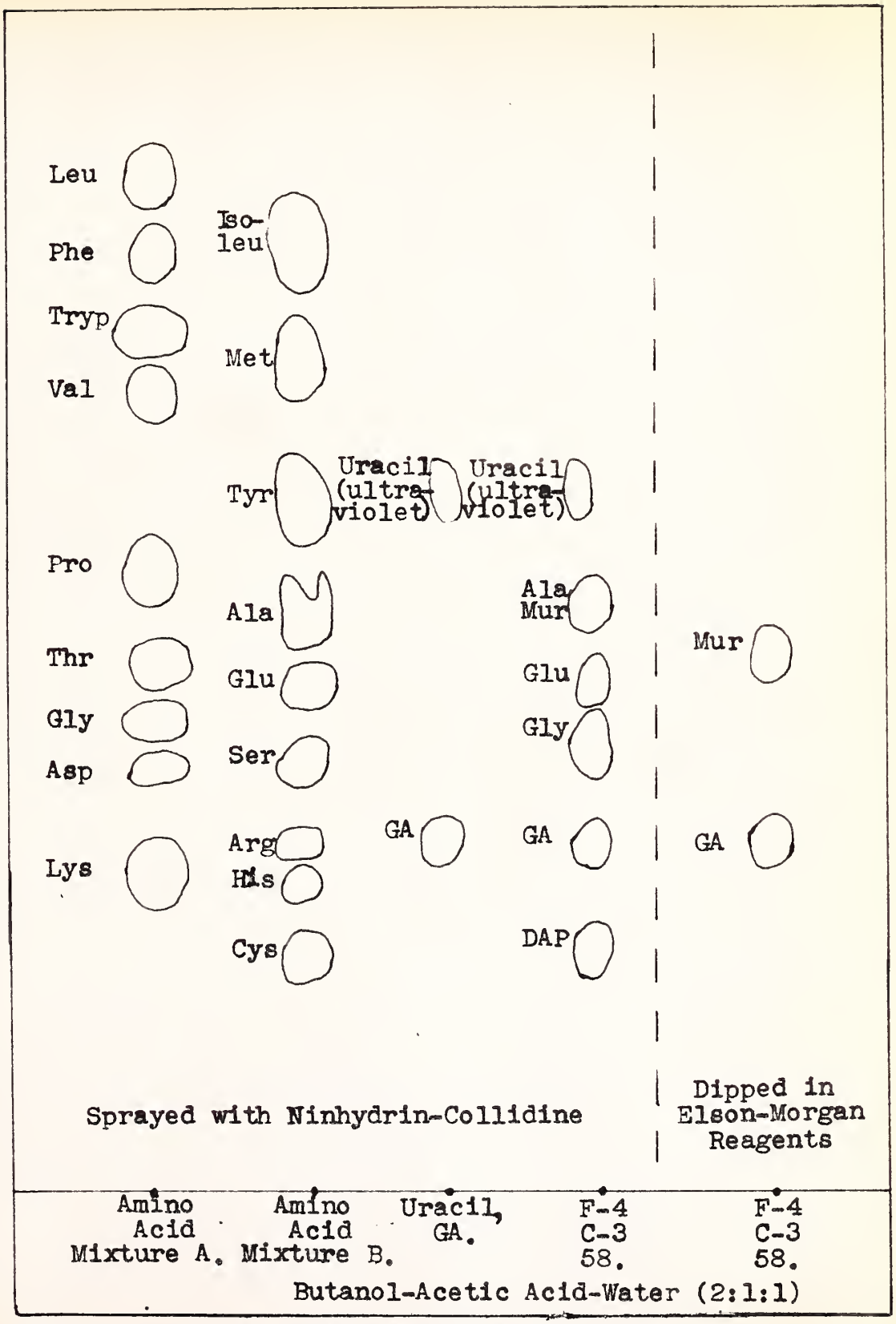


Figure 5. Chromatogram of hydrolyzed normal E. coli nucleotide fraction F-4-C-3-58.

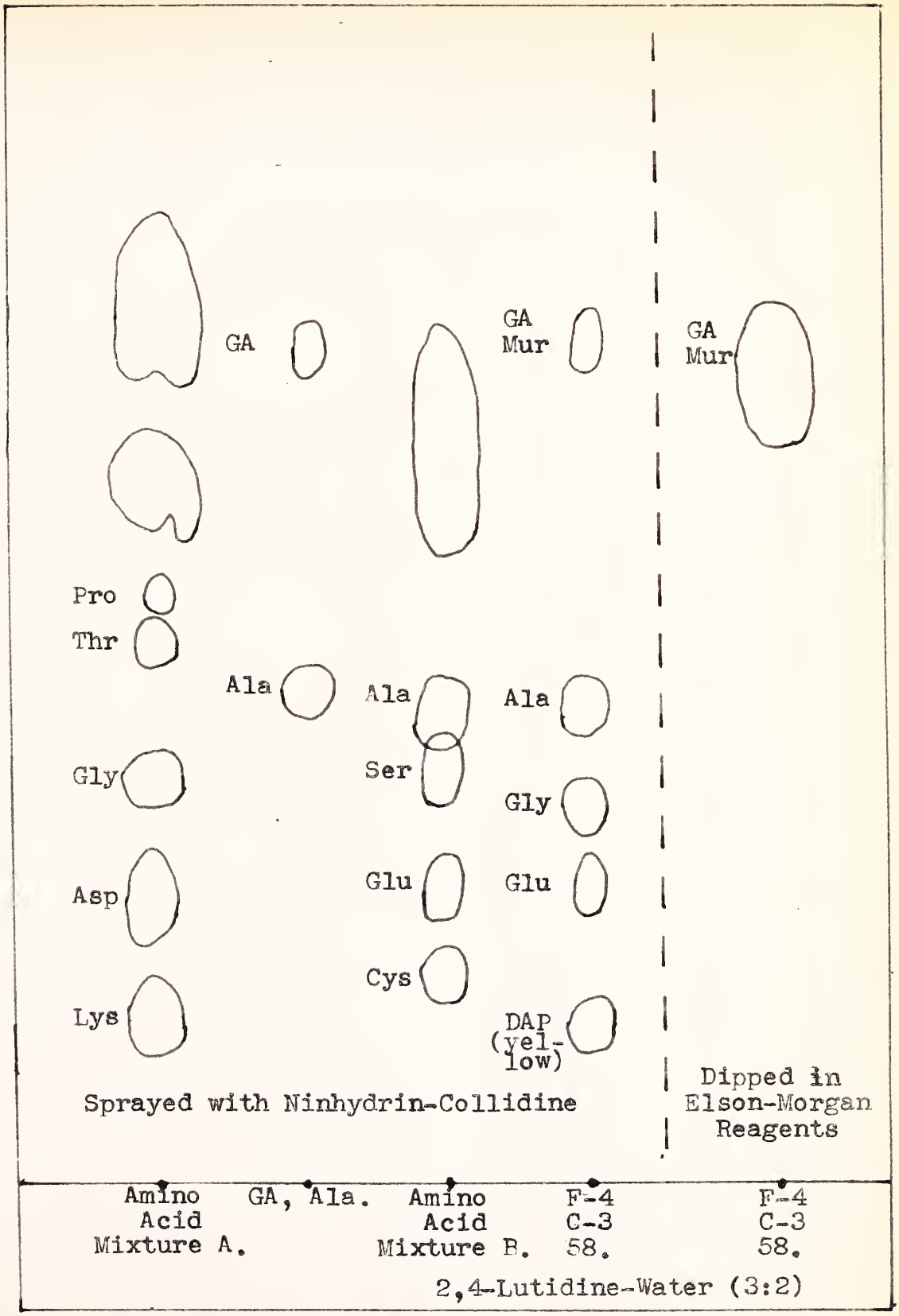


Figure 6. Chromatogram of hydrolyzed normal E. coli nucleotide fraction F-4-C-3-58.

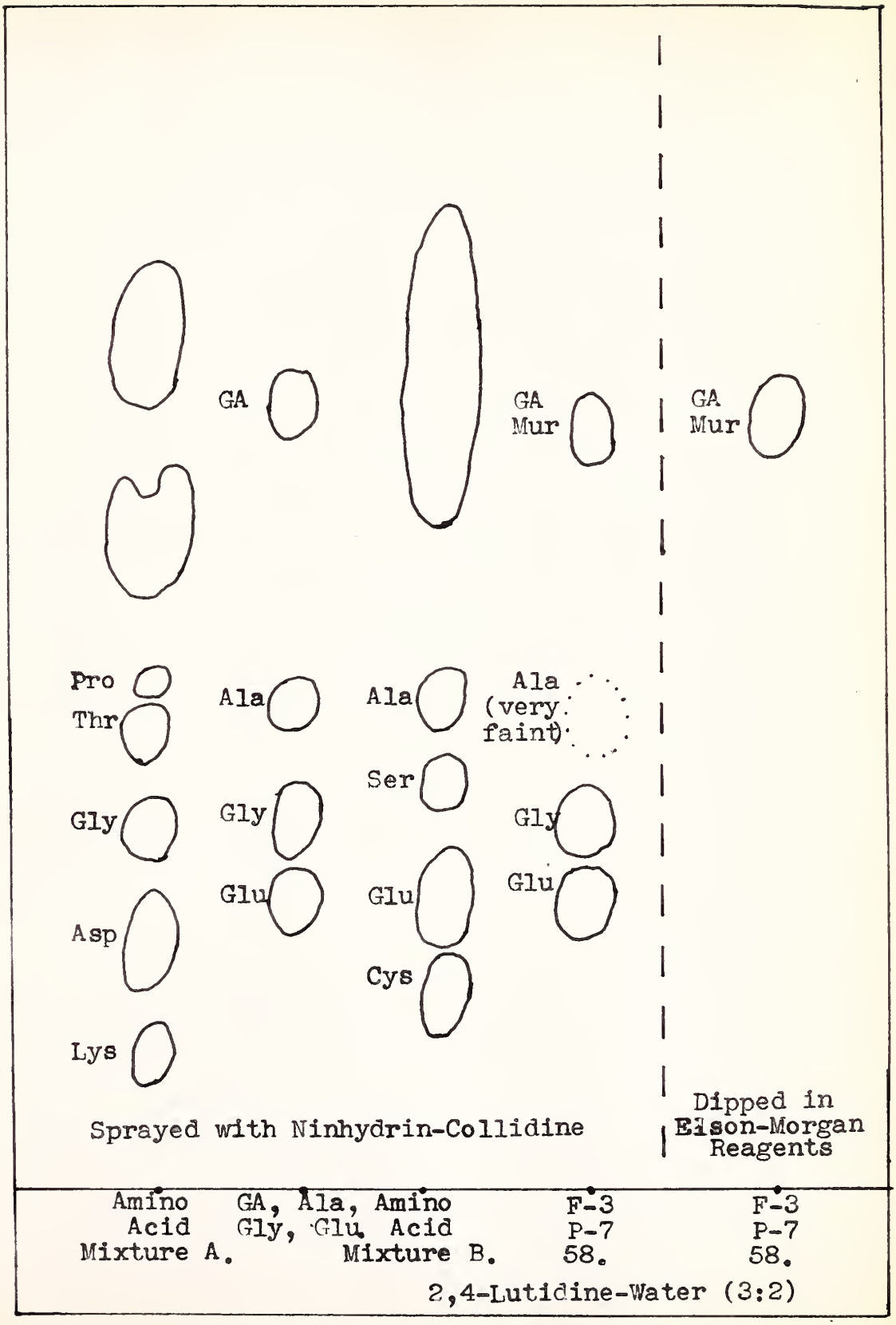


Figure 7. Chromatogram of hydrolyzed protoplast nucleotide fraction F-3-P-7-58.

fractions contained uracil, muramic acid, glucosamine, glutamic acid, glycine and in one case a trace amount of alanine. One of the two fractions from the normal E. coli culture contained considerably larger quantities of alanine and in addition α,ϵ-diaminopimelic acid. The second fraction from this same culture was essentially the same as those of the protoplasts.

An indication of the approximate ratios of some of the fraction components was obtained from the relative intensity of the various spots and from visual comparison with standards on the same chromatograms. The molecular quantities of uracil and amino acids were approximately of the same order of magnitude, whereas muramic acid and glucosamine (which are undoubtedly partially destroyed by the hydrolysis) were present in somewhat smaller quantities. In addition to the substances mentioned very much smaller amounts of unidentified ninhydrin-reacting and ultraviolet-absorbing materials were seen on some of the chromatograms probably as a result of the relative impurity of the nucleotide fractions prior to hydrolysis.

This impurity raises the important question of whether the components of the hydrolysates were originally present in the cell as part of one compound or whether their presence in the same hydrolysate is merely an artifact of the separation procedures. Had the hexosamines and amino acids originally been present as single compounds, they would have been eluted early in the 4 N formic acid section and could not have

contaminated these fractions. They might, however, have been joined in a peptide (perhaps derived from the cell wall). Such a peptide would have had to be consistently eluted from the ion exchange column and from charcoal with the nucleotides. With the large (100 ml.) volumes of ethanol-ammonia used in the charcoal elution, such behavior would be most unlikely. In addition it would have to have the same Rf (less than 0.2) in the butanol-acetic acid as the uracil nucleotides. Inasmuch as the Rf's of similar peptides studies by Park (62) were always greater than 0.4, this is also unlikely. In view of the extreme improbability of this series of coincidences, it would seem that while absolute proof that the components identified were originally part of a single compound is lacking, such a conclusion is extremely likely from the presumptive evidence obtained.

C. The Relative Amounts of the Various Substances in the Protoplasts and Normal Bacteria. It is of interest to estimate and compare the total quantities of acid-soluble nucleotides and the proportion of the various fractions in the protoplasts and normal E. coli. The data of table IV show the total absorption of the nucleotide fraction and the proportion of each fraction characterized in the various cultures. (The cytidylic acid fraction was so poorly separated from the large DPN peaks that no reliable estimation of the amount of this substance could be obtained). As before

TABLE IV

Total and Component Acid-soluble Nucleotides in Normal Cells and Protoplasts

Elution	Total Acid-soluble nucleotides	Total Nucleotides / Protein	DPN / Total Nucleotides	AMP / Total Nucleotides	Amino-acid nucleotides / Total Nucleotides
Protoplasts					
P-1-58	112	0.345	0.180	0.137	-----
P-2a-58	179	0.263	-----	-----	-----
P-2b-58	355	0.374	-----	-----	-----
P-2d-58	219	0.243	-----	-----	-----
P-2-58 (combined)	---	-----	0.387	0.125	-----
P-3-58	262	-----	0.390	0.0487	-----
P-6-58	2,240	0.448	0.312	0.0528	0.042
P-7-58	3,300	0.560	0.320	0.0687	0.026
P-8-58	2,593	0.496	-----	------	0.028
Mean	-----	0.390	0.0318	0.0864	0.032
± S.D.(of mean)	-----	±0.047	±0.053	±0.031	±0.015
Normal Cells					
C-2-57	150	0.324	-----	-----	-----
C-3-57	61.0	0.335	-----	-----	-----
C-4-57	144	0.408	-----	-----	-----
C-5-57	100	0.379	-----	-----	-----
C-1-58	305	-----	0.109	-----	-----
C-2-58	425	0.425	0.094	0.0800	0.081
C-3-58	2,000	0.348	0.164	0.0941	0.113
Mean	-----	0.370			
± S.D.(of mean)	-----	±0.047	0.122 ±0.053	0.0870 ±0.031	0.097 ±0.015
Difference (normal - protoplast)	-----	not significant	0.196 (p=0.01)	not significant	0.065 (p=0.05)
Percent change observed in protoplasts	----	-----	160%	-----	64%
Minimum detectable difference*	-----	33.0%	----	93%	----

*Minimum change that could have been detected (i.e. would have been statistically significant) with observed standard deviation. (p=0.05.)

in all instances where the amount of a substance was the same in the two forms, the minimum difference that could have been detected with the errors encountered has been calculated. In view of results obtained with other organisms it is interesting to note that (using protein content as a basis of comparison) there was no appreciable change in total nucleotide content as a result of protoplast formation.

In both the protoplasts and normal forms the fractions characterized account for slightly less than half the total nucleotide absorption; and of these fractions, DPN (fraction one in the elution diagrams) is clearly the largest component. There is a definite increase in the DPN content in the protoplasts, and the proportion of this substance is approximately two and one half times that of the control rod cells. The adenylic acid contents of the two cell types is approximately the same (within the wide limits of the imprecision of the determination) and it may be seen that DPN and adenylic acid together make up 20 to 35 per cent of the nucleotides in each form.

Unfortunately only meager data are available to estimate the proportion of amino acid containing nucleotides, and in one case a correction had to be made for obvious contamination of the fractions from the column. The hexosamine nucleotides of elution C-3-58 were poorly separated on the column and the ultraviolet absorption of the pooled peaks is quite

obviously increased by impurities. The value in the table is based on the absorption of the fractions after elution from paper with a correction for the average loss in the charcoal step of the corresponding fractions of other cultures. Any error so introduced would tend to decrease the estimate of the proportion of these nucleotides in the normal cells and thus exaggerate any increase that might occur in the protoplast. It should also be noted that the data given reflect only the fractions studied and that there is no assurance that there are not other amino acid or hexosamine nucleotides among the peaks not characterized. With these limitations in mind, it can be seen that in the two forms these nucleotides make up less than 10 per cent of the absorption of the acid-soluble fraction and that there is a significantly smaller amount in the protoplasts. An appreciable increase in proportion of such nucleotides in the penicillin protoplasts is therefore most unlikely.

DISCUSSION

A. The Nucleic Acids and Protein of the Protoplasts and Normal Cells. It does not seem possible to fit all the experimental results described into one framework in terms of the action of penicillin on bacteria. The nucleic acid and protein determinations were done chiefly to serve as a base for comparing the nucleotides of the two forms, and the only conclusion that may be drawn from considering them alone is that there is little or no difference in the ratio of RNA to protein in the protoplasts and normal cells. This agrees with somewhat similar studies (40) of these parameters in round cell formation in Proteus vulgarus. During that process there was a slight increase in total RNA and protein mostly occurring prior to the cessation of cell division, but the ratio of these two substances in the culture remained constant. It is interesting that these observers found that for a short time the rate of increase of total DNA of the culture was greater than that of RNA and protein.

It is difficult to find other experiments which are directly comparable to the present work. Mitchell and Moyle (56) measured some of these parameters in staphylococci treated with sufficient penicillin so that growth (measured by dry weight and turbidity increase) continued at a reduced rate and, perhaps in keeping with the results observed here, found only a slight change in the ratio of total nucleic acid to dry weight. Measurements of nucleic acid and protein have been made in E. coli treated with penicillin (67) but unfortunately in these studies there was leakage of a

relatively large amount of cellular RNA and protein into the medium, and it does not seem meaningful to compare results in such preparations with those in the more stabilized protoplasts. The same is true of the relatively large number of investigations of nucleic acid and/or protein synthesis done in other organisms under a variety of conditions in which cell death and/or frequently partial lysis probably occurred. Probably the most that can be concluded from the results obtained is that they are not inconsistent with those of other observers (40) who suggest that in light of the increase in total and relative amounts of DNA, that DNA synthesis continues while RNA and protein synthesis are inhibited in the stable penicillin round forms of the several species studied (58).

The central question of the validity of the use of total protein of the culture as a base for comparing other parameters is whether the quantity of protein per cell is the same in the normal E. coli and protoplasts. Lederberg (44,46) found that during protoplast formation, total cell number remained constant. During round cell formation in proteus total protein of the culture is also constant (after turbidity increase stops). The almost identical morphologic changes during protoplast formation in E. coli and round form or large body formation in other gram negative species which do not require osmotic stabilization as well as the fact that both protoplasts and round cells are precursors

of L-forms (15,46) make it almost certain that the two processes are analagous and that prior to lysis the resulting rounded cells are probably basically similar. Therefore, it is likely that the quantity of protein per cell does not change significantly during protoplast formation.

B. Origin of the Free Glucosamine of the Hydrolyzed Nucleotide Fractions. The composition of the nucleotides which on hydrolysis yielded uracil, muramic acid, glucosamine, and the three amino acids is not altogether clear from the experimental results. The question of whether glucosamine was present as such in the original nucleotides or was formed during the prolonged strong acid hydrolysis by cleavage of the ether linkage of muramic acid cannot be definitely answered. It is stated in the literature that muramic acid is remarkably stable in the presence of acid (41). However, after 15 hours hydrolysis under similar conditions only 23 per cent of the muramic acid presumed to be present was preserved as such and the remainder could be quantitatively accounted for as ammonia in the hydrolysate solution (62). No mention was made of glucosamine as a product of such hydrolysis. Unfortunately the necessary prior purification and hydrolysis under milder conditions was not done and while it does not seem unreasonable that an ether linkage would be at least partially cleaved under the conditions employed (24), a definite statement cannot

be made. It is possible that glucosamine (or perhaps acetyl glucosamine) was originally a component of the same nucleotide as the amino acids or of another uridine nucleotide (uridine diphosphate actylglucosamine has been isolated from mamalian tissue, yeasts, and the pneumococcus and it might well also be present in E. coli (8,76,77)).

C. The Biochemical Role of the Amino Acid-containing Nucleotides in E. Coli. If one ignores the question of the origin of the free glucosamine of the hydrolysate, nucleotides of composition similar to the hydrolysates found have been definitely isolated in relatively large amounts from staphylococci (62) and a diaminopimelic acid requiring mutant of E. coli (89), and preliminary reports indicate their presence in Streptococcus hemolyticus (51), and Lactobacillus helveticus (4,85). Muramic acid and D-glutamic acid have been found only in the nucleotides, in bacterial cell walls, and in certain hexosamine containing peptides of spores which are probably derived from the cell wall (97). (In addition, a phosphate ester of muramic acid has recently been isolated in very small amounts from the precipitated proteins of L. helveticus (4) and E. coli (3)). This as well as the fact that these substances and other amino acids are present in the same ratio in the cell wall and the nucleotides (64), makes it quite likely that uracil nucleotides of this type are coenzymes in cell wall synthesis in gram positive bacteria. The cell walls of gram negative organisms, however,

have for a long time been known to contain lipids and a large number of other amino acids in addition to the few present with the hexosamines in the gram positive cell wall (53,97). Recently, however, it has been shown that the walls of gram negative bacteria are in reality probably composed of two layers: an outer phenol-soluble shell of lipoprotein containing all the common amino acids and an inner peptide-polysachride layer resembling the gram positive cell wall and containing glucosamine, muramic acid, alanine, glutamic acid, glycine, and lysine (95,96). This finding makes it quite probable that the nucleotides isolated from the normal cells and protoplasts are also coenzymes in cell wall synthesis.

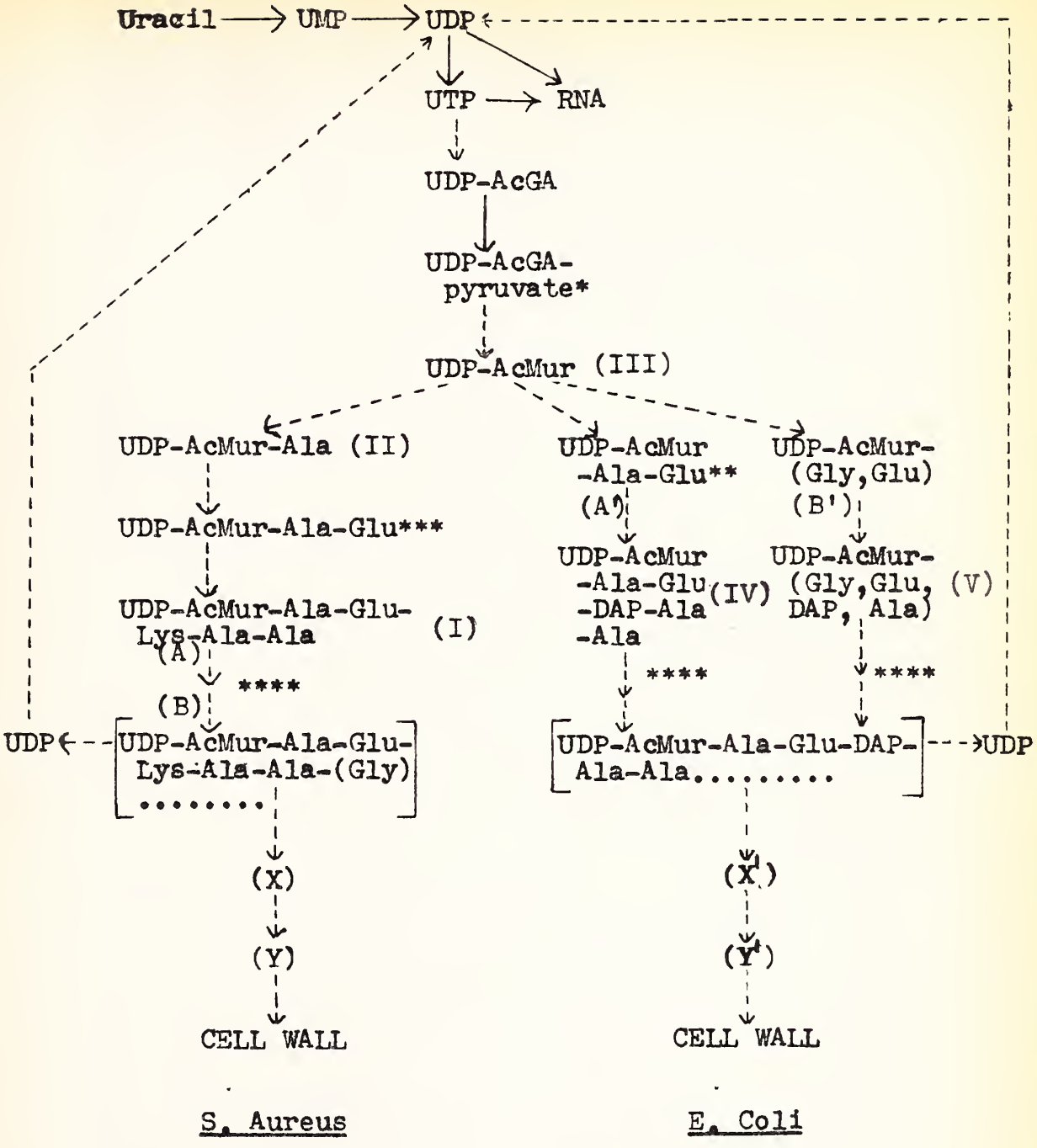
D. The Cell Wall Lesion in the Protoplasts. The nature of the cell wall of the penicillin protoplasts has been clarified by several recent investigations. The finding that E. coli treated with penicillin in hypertonic sucrose continue to incorporate radioactive glucose into the cell wall at the same rate as normal bacteria and that under such conditions the wall shows no discernable morphologic change (69) make it apparent that there is no total inhibition of cell wall synthesis but rather alteration in the chemical structure to procure the changes seen in the protoplasts. Weidel (95,96) has observed that organisms from which the outer lipoprotein layer has been removed by phenol retain their rod shape and osmotic impermiability, two properties

which are conspicuously absent in the protoplasts. As he suggests, it is probable that it is the inner hexosamine-containing layer which is affected in protoplast formation. The well known penicillin sensitivity of gram positive bacteria which contain only this polysaccharide-peptide layer of the wall would also be in keeping with the view that it is this layer whose synthesis is affected by the drug. Further evidence for this concept comes from the demonstration that mutants unable to synthesize one of the chief components of this layer, diaminopimelic acid, undergo swelling and lysis in deficient medium. If protected with hypertonic sucrose, they will develop into round forms apparently quite similar to the penicillin protoplasts which regain the rod shape and divide on addition of diaminopimelic acid (6,54). In addition, direct analyses of the cell walls of penicillin round forms of V. metchnikovi (73) showed a decrease in hexosamine content, and similarly in E. coli penicillin was found to inhibit selectively the incorporation of diaminopimelic acid into the cell wall while incorporation of radioactive glucose (presumably into the outer lipoprotein layer) continued virtually unchanged (57).

E. The Influence of Penicillin on the Amino Acid Nucleotides. Since the penicillin-induced lesion in the protoplasts is, therefore, almost certainly in the layer containing the components of the nucleotide fractions studied in these

experiments, it is interesting to consider the step or steps in nucleotide metabolism and cell wall formation which might be affected by the drug. Present knowledge of the mechanisms of nucleotide formation and utilization in cell wall synthesis in E. coli and S. aureus is summarized in figure 8. Unfortunately almost nothing is known about the mechanisms by which the muramic acid-peptide moiety combines with other cell wall constituents, passes through the outer membrane of the bacterium and becomes incorporated into the wall polymer, and the representation of these steps is, therefore, highly diagrammatic. While it is therefore not possible to delineate the specific biochemical reaction interfered with by penicillin, one can on the basis of evidence from these experiments and those of other workers suggest the stages in cell wall synthesis and the types of reactions that might be inhibited.

The finding that although one of nucleotide fractions from the normal bacteria contained DAP, this amino acid was not present in any of the four protoplast fractions from the three cultures suggests that in E. coli penicillin may inhibit the incorporation of diaminopimelic acid into a uridine nucleotide. This would imply that the drug alters cell wall synthesis by inhibiting the formation of nucleotide precursors (steps A' and B' in the figure) rather than by inhibiting the incorporation into the wall of the groupings



* (88). **(89). ***(87). ****Number and nature of steps are uncertain.

Figure 8. Pathways in the formation and utilization of uridine nucleotides in cell wall synthesis in E. coli and S. aureus. The evidence for various intermediates and their signifigance in relation to the action of penicillin are discussed in the text. Steps represented with broken arrows are likely but not established. Compounds and portions of compounds in brackets and parentheses are not isolated or of uncertain structure. Ac--acetyl; other abbreviations as in figures 5-7 (page 22).

from such precursors once they are formed. If this concept is correct, the decrease in DAP incorporation into the wall (57) (noted above) would be a consequence of impairment of formation of a DAP-containing uridine nucleotide. It should be emphasized that because the nucleotide fractions in the normal cells and protoplasts were only crudely purified and not definitely characterized, the inhibition of formation of a DAP-containing nucleotide, although suggested by the evidence presented, is certainly not definitely proven. If the same type of mechanism is operative in S. aureus, one would have to assume that there exist uridine nucleotide precursors beyond those which accumulate in the presence of penicillin (I, II, III in the figure). The accumulation would then be due to loss of ability to form these more advanced precursors (impairment of reactions A and B). Although the nature of these more advanced precursors is unknown, it is interesting that uridine nucleotide fractions containing aspartic acid and glycine in addition to the constituents of (I) have recently been detected in S. aureus (39). Since these two amino acids (as well as glucosamine) are found in the cell wall (38) the existence of nucleotide precursors beyond the stage of (I) is not an unreasonable assumption. Conformation by actual characterization of such compounds from the non-inhibited staphylococcus is certainly necessary.

A uridine nucleotide containing muramic acid, alanine,

glutamic acid and DAP (IV in the figure) has recently been isolated by Strominger (89) from a DAP-requiring mutant of E. coli. Assuming that this compound (which did not contain glycine) is also present in the strain used in these experiments, several mechanisms might be proposed. Formation of (IV) might have been interfered with in the protoplasts. The compounds containing glycine and glutamic acid found in these forms would then be an entirely unrelated nucleotide. The compound with glycine, glutamic acid, alanine, and diaminopimelic acid (V) detected here in the normal E. coli could then have arisen either independently or perhaps from some combination of groups of the protoplast nucleotide with glycine and glutamic acid and (IV). Mechanisms of this type are of course extremely speculative, but it is interesting that the only fraction definitely found to contain alanine was the one from the intact cells (V) which also contained DAP. This quite tentative evidence does suggest that alanine and diaminopimelic acid are perhaps incorporated into a nucleotide as a previously formed grouping. It should be realized, however, that there is no other evidence to support this concept and that such mechanisms of nucleotide interaction have not been established.

Although further speculative mechanisms could undoubtedly be proposed, it is obvious that more precise knowledge of the pathways of cell wall formation from the uridine

nucleotides and of the effect of penicillin on the steps involved is needed. A fairly large number of amino acid nucleotides have been detected in different bacterial species in both normal and penicillin blocked cells; and, when the structures of more of these compounds are established mechanisms can be more precisely formulated. Undoubtedly a great deal could be learned from studies of the synthesis and interconversion of these compounds in cell free systems and of the effect of penicillin on the reactions involved. (It might, however, be found that the antibiotic did not block in cell free systems the reaction which it affects in the whole bacterium if, for example, it, as proposed recently (26), acts by blocking formation of an enzyme involved in cell wall synthesis rather than by interference with the action of the preformed enzyme.)

Another method of testing the hypothesis that penicillin acts by blocking a step in the formation of the uridine nucleotides involved in cell wall synthesis, would be to study the effect of the antibiotic or synthesis of "colominic acid", a polymer composed of neuraminic acid and the amino acids found in cell walls (5). This substance which may function as an agglutination inhibitor in the E. coli strain which produces it (5) has recently been shown probably to be formed via a mechanism involving high molecular weight peptide neuraminic acid polymers containing UDP (59). If penicillin were found to interfere with such a synthesis, it would be

strong evidence that its action on cell wall formation involved inhibition of some portion of the pathway utilizing the uridine nucleotide coenzyme. (The alternative would be that the drug acted on later stages involving intermediates (represented diagrammatically in figure 8 as X and Y) formed after the splitting of the muramic acid peptide from the UDP moiety.)

* * *

The striking difference between the findings in penicillin inhibited sensitive S. aureus and the protoplasts studied in these experiments is the lack of accumulation a uridine hexosamine nucleotide in the protoplasts even though it is probable that penicillin also in E. coli acts by interfering with formation or utilization of these nucleotides. In the two sensitive organisms studied by Strominger (85) (L. helveticus and S. aureus) an increase in hexosamine nucleotides did occur. However, in the resistant species (S. fecalis and a resistant strain developed from the previously used S. aureus) no increase was observed even at penicillin concentrations sufficient to kill these strains. It may be, therefore, that the findings in the protoplasts are similar to those in the gram positive resistant species. The mechanism of uridine hexosamine nucleotide accumulation might thus be related to

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3. A preliminary report states that the hexosamine content of the acid-soluble fraction is increased in E. coli when growth is inhibited with 6-azauracil (90). Identification of the yet uncharacterized compounds responsible for this increase would perhaps help elucidate the pathways of cell wall synthesis and possible sites of action of penicillin.

penicillin resistance in the species studied. (Since the resistant S. aureus strain used was laboratory produced, it presumably acquired resistance through some mechanism other than the production of penicillinase (1)). Comparison of the amino acid nucleotides of the normal and penicillin inhibited forms of gram positive resistant organisms to ascertain whether there are not changes similar to those seen in the protoplasts would obviously be desirable.

The biochemical mechanisms responsible for nucleotide accumulation in sensitive but not resistant species are obscure. Although other more complex theories could be proposed it may simply be that the quantity of uridine nucleotides is normally higher in resistant bacteria and hence the increase in a precursor which might be produced by penicillin is not detected. An approach to the problem would be to study the formation of the muramic acid nucleotides and elucidate the mechanism of the enormous (25 fold) increase that takes place in the inhibited sensitive staphylococcus. In the course of utilization of these compounds in cell wall synthesis, the UDP moiety must be split from the hexosamine-peptide and is probably reutilized. The relationship of the small amount of peptide nucleotide present in the cell normally to the amount formed and utilized during the course of cell division is unknown, but it is possible that the huge quantity found in penicillin inhibition represents the total amount involved in the formation of one new cell wall.

The experiments done with radioactive uracil have demonstrated that penicillin greatly increases the proportion of added isotope incorporated into the uridine nucleotides (86) but obviously further work is needed to elucidate the normal turnover of the nucleotides in cell wall formation and clarify the mechanism of the tremendous penicillin induced increase.

F. The DPN Content of Protoplasts and Normal Cells.

A striking difference in the nucleotides of the protoplasts and normal cells was the marked increase in the proportion of DPN in the protoplast nucleotides. A similar increase was found in Lactobacilli treated with chloramphenicol[4], but no increase was reported in the penicillin inhibited staphylococcus under conditions leading to uridine nucleotide accumulation (85). The mechanism of DPN increase in the protoplasts is not clear. It may be a general phenomenon occurring in a number of bacteria inhibited by various agents: perhaps the metabolic pathways employed under such conditions utilized this coenzyme at an increased rate and in response, an increased amount is synthesized. Since no increase seemed to occur in the sensitive staphylococcus treated with penicillin, it is possible that the increase in the protoplasts may be a consequence of the different metabolic pathways utilized by the resistant organisms in the presence of the drug. Study of the effect of penicillin on the relative DPN content of the gram

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4. Carter, C.E. and Felsenfeld, H., personal communication

positive resistant bacteria would help decide this point and might also aid in elucidation of the mechanism of penicillin resistance.

SUMMARY

Uridine nucleotides containing muramic acid and amino acids found in the cell wall are known to accumulate in penicillin-inhibited sensitive bacteria. It has been demonstrated furthermore that in concentrated sucrose medium the antibiotic induces the development in E. coli of osmotically unstable spherical forms known as protoplasts. These findings suggested that penicillin interferes with formation of the polysacchride-peptide layer of the cell wall. This and other evidence that the drug alters bacterial nucleotide metabolism prompted the present study of the acid-soluble nucleotides, protein, and nucleic acids of the penicillin protoplasts and the rod-shaped organisms from which they are derived.

Using protein content of the culture as a basis of comparison, no appreciable difference was found in the RNA and total nucleotide content of the protoplasts and normal bacteria. Relative to total acid-soluble ultraviolet absorption the DPN content of the protoplasts was two and one half times that of the rod-shaped organisms.

Both protoplasts and intact bacteria yielded fractions which on prolonged acid hydrolysis were found to contain uracil, muramic acid, glutamic acid, glycine, and glucosamine (the latter perhaps as a product of the cleavage of the ether linkage of muramic acid). The normal E. coli, however, yielded as well a second fraction containing in addition to these substances, alanine and α,ϵ-diaminopimelic acid, a component not found in any of the hydrolyzed nucleotides from the protoplasts. These amino acid nucleotide fractions had an ultraviolet absorption less than 10 percent of the total of the acid-soluble fraction and were not increased in the protoplasts.

These findings suggest that in altering cell wall formation, penicillin acts by preventing the incorporation of components such as diaminopimelic acid into nucleotide precursors rather than (as might have been supposed) by interfering with utilization of these precursors once they are formed. Further work is needed to establish the structures of the compounds producing the fractions enumerated and to test this proposed concept in both sensitive and resistant bacteria. The lack of accumulation of any nucleotide cell wall precursor in the protoplasts is in accordance with results in resistant gram positive organisms and may be related to mechanisms of penicillin resistance.

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