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The Phosphorus-containing Compounds of Gram-positive and Gram-negative Organisms in Relation to the Gram Staining Reaction

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SUMMARY: The phosphorus-containing compounds of certain Gram-positive and Gram-negative organisms were estimated by a modification of the Schmidt & Thannhauser (1945) procedure. Another method (Mitchell & Moyle, 1950, 1951*a, b*, 1954) in which pentosenucleic acid (PNA) was estimated from the optical absorption of the 'PNA fraction' at 260 m μ . was found to be inaccurate, because of the presence of ultraviolet-absorbing impurities. A more accurate method for estimating PNA has now been developed, in which the nucleotides are separated from impurities by paper electrophoresis. The results thus obtained show that non-nucleotide phosphorus-containing compounds are present in the 'PNA fraction' of both Gram-positive and Gram-negative organisms. There is, therefore, no evidence to connect these compounds with the Gram staining properties of Gram-positive micro-organisms (cf. Mitchell & Moyle, 1950, 1951*a, b*, 1954).

It has been shown that magnesium ribonucleate, in association with certain proteins and possibly polysaccharides, is the complex responsible for the retention of the Gram stain in Gram-positive micro-organisms (Henry & Stacey, 1943, 1946; Henry, Stacey & Teece, 1945; Jones, Muggleton & Stacey, 1950). These conclusions, which have been largely confirmed by other workers (Bartholomew & Umbreit, 1944) have since been questioned by Mitchell & Moyle (1950, 1951*a, b*, 1954) who claimed that Gram-positive organisms contain a particular phosphoric ester fraction (XSP) which is absent from Gram-negative species and that this 'XSP factor' is responsible for the retention of the stain in the Gram reaction. In view of these claims, it was decided to re-investigate the problem and in particular to analyse various micro-organisms for their XSP content.

METHODS

Organisms used and methods of cultivation. The following organisms were used: (a) Gram-positive; baker's yeast (Distillers Co.), *Bacillus subtilis*, *Clostridium welchii*, *Sarcina lutea*, *Streptococcus faecalis* (NCTC, 370); (b) Gram-negative; *Aerobacter aerogenes* (NCTC 8172), *A. cloacae* (NCTC 8813), *Escherichia coli* and *Klebsiella pneumoniae* (NCTC 3279). They were grown in a medium containing (% w/v): 0.5, Lab Lemco; 1, Oxoid bacteriological peptone; 1, glucose; 0.5, sodium chloride. The solution of Lab Lemco, peptone and sodium chloride was adjusted to pH 9, boiled for 5-10 min. and the resulting precipitate filtered off. The filtrate was adjusted to pH 7.5 and the solution autoclaved at 10 lb./sq.in. for 15 min. Sterile glucose solution was then added aseptically. The complete medium was inoculated with an

18 hr. culture of the required organism. The cultures of *Aerobacter aerogenes*, *A. cloacae*, *Bacillus subtilis* and *Sarcina lutea* were aerated during growth. After incubation at 37° for 18 hr. (except for *S. lutea* which was grown for 48 hr.) the organisms were centrifuged down, washed with 0.85% (w/v) sodium chloride and freeze-dried. The *Klebsiella pneumoniae* cultures were autoclaved at 15 lb./sq.in. for 10 min. before centrifugation.

Determination of the phosphorus-containing compounds. The determination of phosphorus-containing compounds was initially carried out by Mitchell & Moyle's (1951*a*, *b*, 1954) method (summarized in Fig. 1), which was an adaptation of the Schmidt & Thannhauser (1945) procedure, thus giving an estimate of the following fractions: acid-soluble inorganic phosphorus (AI); acid-soluble organic phosphorus (AO); lipid phosphorus (L); pentosenucleic acid phosphorus (PNAP); deoxypentose nucleic acid phosphorus (DNAP); phosphoprotein phosphorus; XSP phosphorus. Phosphorus was determined as described by Jones, Lee & Peacocke (1951). After extraction of the lipids the organisms were dried *in vacuo* over silica gel instead of over phosphorus pentoxide in order to eliminate any possibility of contamination with the latter. The XSP phosphorus was obtained by subtracting from the total phosphorus of the 'PNA fraction' the phosphoprotein phosphorus (estimated as the inorganic phosphorus of the PNA fraction) and the PNA phosphorus, the latter being calculated from the optical extinction of the solution at 260 m μ . For this purpose Mitchell & Moyle assumed that the absorption of other components at 260 m μ . was negligible and that the ϵ_p value of all the pentose nucleic acids was 10,000 under the conditions of the estimation. ($\epsilon_p = 30.98 E/cl$, where E = extinction of the solution; c = concentration of phosphorus in g./l.; l = thickness of absorbing layer in cm.; Chargaff & Zamenhof, 1948.)

It became obvious, however, that this method of estimating PNA was inaccurate, both due to the fact that absorption at 260 m μ . of components other than nucleotides was far from negligible and that the ϵ_p values of PNA vary according to the source and are not equal to 10,000. The PNA was, therefore, subsequently determined by separation of the nucleotides of the PNA fraction by paper electrophoresis on Whatman No. 3 paper at pH 3.5 for 8.5 hr. at 1400 volts by Crosbie, Smellie & Davidson's (1953) method as previously described (Foster, Jones & Rizvi, 1956). The nucleotides were eluted from the paper with phosphate buffer (pH 7) and estimated spectrophotometrically. With pure yeast PNA 95% recovery was obtained. When applied to the PNA fractions of the micro-organisms, good separation of the nucleotides was not obtained, however. Since this was thought to be due to the presence of salts, N-KOH was used to digest the organisms instead of N-NaOH, and perchloric acid to precipitate DNA at pH 1 instead of trichloroacetic and hydrochloric acids. After separating the DNA, protein, and potassium perchlorate precipitates by centrifugation, the supernatant liquid was adjusted to pH 4 and freeze-dried. The nucleotides were then extracted from the freeze-dried material with 60% (v/v) ethanol in water and applied to the paper. With pure yeast PNA, 95% recovery was obtained. This showed that there was a negligible loss of nucleotides on the potassium perchlorate precipitate (cf. Elson, Trent

& Chargaff, 1955). The non-interference of other cell constituents in the separation was established by estimating the nucleotide content of yeast PNA; in presence and in absence of a yeast extract. Satisfactory separation of the nucleotides was obtained and the recovery of PNA was again 95%. Later work showed that the analysis could be improved in the following respects. (1) It was not necessary to adjust the pH value of the solution of nucleotides from pH 1 to pH 4 in order to achieve separation. (2) Whatman No. 1 paper could be used instead of Whatman No. 3, thereby decreasing the ultraviolet absorption of the blanks and simplifying the elution of the nucleotides. (3) Freeze drying of the PNA fraction and subsequent extraction of the nucleotides with 60% (v/v) ethanol in water was not necessary if, after placing the ionophoretogram on the glass plates in the usual manner, it was left for 15 min. before passing the current. This allowed all the material to dissolve in the buffer and so prevented undissolved fragments of the PNA fraction from interfering in the separation of the nucleotides. (4) More rapid and complete extraction of the nucleotides from the paper was obtained by the use of 0.1N-HCl at 37° for 18 hr. instead of phosphate buffer of pH 7. Degradation of the nucleotides was negligible and the recovery of PNA was in this case 97–100% of the theoretical. The XSP content was then determined using this more accurate value for the PNA phosphorus. The final procedure is shown in Fig. 1.

RESULTS

Distribution of phosphorus-containing compounds in micro-organisms

A number of Gram-positive and Gram-negative micro-organisms were analysed by the methods described above. The results (Table 1) show several marked differences from those obtained by Mitchell & Moyle. Using the method of these authors for calculating the XSP content, the distinction between Gram-positive and Gram-negative organisms was not nearly so marked as the earlier results (Mitchell & Moyle, 1954) suggested. Thus, although, in general, the Gram-positive organisms appeared to contain more XSP factor than did the Gram-negative species, one or two anomalies were found; *Klebsiella pneumoniae* appeared to have a higher XSP content than did baker's yeast and *Aerobacter aerogenes* had an appreciable 'negative XSP content'.

From the results obtained by using the electrophoretic method for the estimation of PNA, it was obvious that all the organisms had an appreciable XSP content and that the low values obtained for Gram-negative organisms by Mitchell & Moyle's method were due mainly to the presence of non-nucleotide, ultraviolet-absorbing material in the PNA fraction and, to a less extent, to the use of an inaccurate ϵ_p value for the estimation of PNA. The ϵ_p^{260} values for the PNA from various micro-organisms are shown in Table 2.

Non-nucleotide ultraviolet-absorbing components

The ultraviolet (u.v.) absorption spectra of the PNA fractions from all the organisms studied were not typical of pure ribonucleotides, the absorption at 220 m μ . being much higher than that at 260 m μ . (Figs. 2 and 3 show curves

Table 1. *Distribution of phosphorus-containing compounds in certain micro-organisms*

Code for fractions: L=Lipid phosphorus; AI = acid-soluble inorganic phosphorus; AO = acid soluble organic phosphorus; DNAP = DNA phosphorus; PNAP = PNA phosphorus; XSP = 'Excess phosphorus' in the PNA fraction.

Methods of determination: (a) = determined by Mitchell & Moyle (1954); (b) = determined by Mitchell & Moyle's (1951 a, b, 1954) method; (c) = determined by the present method.

Organism	Gram reaction	μmole phosphorus/g. freeze-dried organism. Fraction																		XSP (% total P)	Non-nucleotide u.v.-absorbing material (% of PNA)*	
		Total	L						AI	Method of determination						DNAP	PNAP	XSP				
			(a)	(b)	(c)	(a)	(b)	(c)		(a)	(b)	(c)	(a)	(b)	(c)			(a)	(b)			(c)
<i>Sarcina lutea</i>	+	411	411	6-12	6-12	44-1	44-1	62-9	62-9	35-0	35-0	178	105	86-9	158	39-3	37-0					
<i>Bacillus subtilis</i>	+	1157	734	7-30	7-30	70	98-6	98-6	238	54-0	54-0	71	58-0	58-0	291	292	188	471	22-4	338	45-8	31-5
<i>Clostridium welchii</i>	+	959	698	8-12	8-12	37	55-0	55-0	30	41-6	41-6	130	56-2	56-2	491	504	304	226	32-7	233	33-0	34-2
<i>Streptococcus faecalis</i>	+	1297	685	26	29-8	146	62-2	62-2	266	87-0	87-0	168	65-4	65-4	459	406	278	232	34-5	162	23-6	28-6
		1047		29		200			129			111			417			161				
Baker's yeast	+	352	328	9-4	24-0	47	71-4	71-4	45	19-3	19-3	22	20-6	20-6	169	169	130	60	24-3	63-6	19-3	19-0
<i>Klebsiella pneumoniae</i>	-	450	450	40-0	40-0	8-0	8-0	5-8	5-8	38-0	38-0	328	207	31-0	152	34-6	28-2					
<i>Aerobacter aerogenes</i>	-	594	459	62	47-7	47-7	50	20-7	20-7	46	70-1	70-1	26-8	26-8	407	317	204	4	-22-1	91-0	19-8	30-6
<i>A. cloacae</i>	†	601	601	67-0	67-0	57-0	57-0	59-0	59-0	41-0	41-0	366	210	366	210	210	210	12-0	167	26-8	37-4	
	†	578	578	68-0	68-0	47-0	47-0	53-0	53-0	45-0	45-0	210	210	210	210	210	210	155				
<i>Escherichia coli</i>	-	664	543	543	63	45-3	45-3	26	13-1	42	49-0	49-0	180	30-0	345	410	223	8	-4-7	182	31-8	43-0
	†	540	540	48-0	48-0	17-6	17-6	49-0	49-0	28-0	28-0	224	224	224	224	224	224	172				

* Assuming that this material has the same optical absorption at 260 mμ as has PNA.

† DNA precipitated with trichloroacetic and hydrochloric acids.

‡ DNA precipitated with perchloric acid.

* In the present investigation a difficulty was encountered in determining the optical absorption of the PNA fraction due to the use of trichloroacetic acid as a precipitant for the DNA. Trichloroacetic acid has an appreciable absorption at 260 m μ . for which it was difficult to correct due to the absorption of an unknown quantity of trichloroacetic acid on the DNA and protein precipitate. In order to eliminate this uncertainty the PNA fraction was extracted four times with ether before the optical density was determined. A blank was obtained by similarly extracting a solution of trichloroacetic acid and hydrochloric acid of the same strength was used to precipitate the DNA. There is no indication of how the blank was determined by Mitchell & Moyle.

Fig. 1. Procedures used for the determination of the phosphorus-containing compounds in micro-organisms.

for two of the organisms; the others were similar.) This indicated the presence of other u.v.-absorbing components in this fraction. These could be separated from the nucleotides by paper electrophoresis at pH 3.5, when the nucleotides migrated to the anode and the other components either remained on the starting line or migrated to the cathode (Pl. 1, fig. 1). These components were eluted, combined, and their u.v.-absorption spectra determined. The spectra (Figs. 2, 3) were similar for the components from all the organisms and showed

no definite peak. This absorption contributed on the average about 30% to the total absorption of the PNA fraction at 260 m μ . (Table 1).

Table 2. ϵ_p^{260} values at pH 1 of PNA from various micro-organisms

Organism	ϵ_p^{260}	Organism	ϵ_p^{260}
<i>Bacillus subtilis</i>	10,600	<i>Aerobacter aerogenes</i>	10,800
<i>Clostridium welchii</i>	10,900	<i>A. cloacae</i>	10,900
<i>Sarcina lutea</i>	10,600	<i>Escherichia coli</i>	10,500
<i>Streptococcus faecalis</i>	10,450	<i>Klebsiella pneumoniae</i>	10,900
Baker's yeast	10,600		

An alternative method for the isolation of these components was to freeze dry the PNA fraction and extract the nucleotides with 60% (v/v) ethanol in water. Most of the non-nucleotide u.v.-absorbing material remained insoluble. This insoluble material gave a positive ninhydrin reaction and a negative reaction for carbohydrate, and therefore appeared to be mainly peptide in nature. This was confirmed by hydrolysing the PNA fraction of *Klebsiella pneumoniae* with 5N-HCl and separating the products by paper electrophoresis at pH 5.0. Upon spraying the paper with ninhydrin, spots corresponding to basic, neutral and acidic amino acids were obtained.

Fig. 2

Fig. 3

Figs. 2, 3. Ultraviolet absorbing components of the PNA fractions of *Klebsiella pneumoniae* (Fig. 2) and of *Bacillus subtilis* (Fig. 3). ●—●, Total PNA fraction; ×—×, Non-nucleotide components; ▲—▲, nucleotides.

The 'XSP factor' of *Klebsiella pneumoniae*

Further evidence for the presence of non-nucleotide phosphorus-containing compounds (XSP) in *Klebsiella pneumoniae* was obtained by the use of radioactive phosphorus (^{32}P). The organisms were grown for 18 hr. at 37° in 500 ml. medium containing 3 millicuries (mc.) of ^{32}P as inorganic phosphate. The culture was then autoclaved, and the organisms centrifuged off, washed with 0.85% (w/v) sodium chloride and freeze dried. The PNA fraction was then prepared as usual and subjected to paper electrophoresis on Whatman No. 3 MM

paper at pH 3.5 for 17.75 hr at 900 V. The u.v.-absorbing compounds were detected in the usual way and the compounds containing ^{32}P detected by autoradiography. The results (Pl. 1, fig. 2) show that in addition to the four nucleotides there were present at least ten other phosphorus-containing compounds, some of which coincided with some of the non-nucleotide u.v.-absorbing components. Since this electrophoretogram was run for a considerably longer time than that shown in Pl. 1, fig. 1, some of the non-nucleotide u.v.-absorbing components had run off the end of the paper.

Some of the components of the XSP factor of *Klebsiella pneumoniae* were isolated and partly identified as follows (cf. Mitchell & Moyle, 1951*a*). The PNA fraction was prepared as previously described (without the use of ^{32}P) from 40 mg. freeze-dried organisms. This fraction was adjusted to pH 9 with ammonia, 95% (v/v) ethanol in water added (1 vol.) and the mixture allowed to stand at room temperature for 2 days. The precipitate (non-nucleotide u.v.-absorbing material) was centrifuged off and to the supernatant liquid there was added M-BaCl₂ solution (0.5 ml.). The precipitate thus formed was centrifuged off, washed twice with 60% (v/v) ethanol in water, extracted with 0.1 N-H₂SO₄ and the precipitate of barium sulphate centrifuged off. The supernatant liquid was adjusted to pH 5 with barium hydroxide solution and the barium sulphate precipitate centrifuged off.

In order to remove nucleotides from the supernatant liquid a charcoal slurry (5 mg. charcoal washed by the method of Lipkin, Talbert & Cohn, 1954, in 1 ml. distilled water) was added dropwise until all the nucleotides had been adsorbed. This was shown by the absence of a peak at 260 m μ . in the optical absorption curve of the solution after the removal of the charcoal. The resulting solution contained non-nucleotide u.v.-absorbing material, a small quantity of inorganic phosphate and 64% of the XSP factor originally present in the PNA fraction. By a similar procedure, 63% of the XSP factor of *Aerobacter cloacae* was obtained. The solution obtained from *Klebsiella pneumoniae* was freeze-dried and the components separated by paper chromatography by techniques described by Benson *et al.* (1951) and Hanes & Isherwood (1949).

Acid-washed Whatman No. 1 and No. 4 papers were used. The phosphates were detected by the use of the spray reagent described by Hanes & Isherwood (1949). After spraying, the papers were dried, heated at 80° for 10 min. and then exposed to u.v. radiation for 10 min. as recommended by Bandurski & Axelrod (1951). After irradiation most of the organic phosphates appeared immediately as blue spots but some only appeared after some time (in one case 12–15 hr.).

When chromatograms were developed on Whatman No. 1 paper with *n*-propanol + water + ammonia (sp.gr. 0.880); (60:10:30), for 18 hr., five spots of R_F values 0, 0.11, 0.26, 0.38, and 0.67 were detected. When a mixture of *isopropanol* + water + ammonia (sp.gr. 0.880), (70:6:30), was used as the solvent on Whatman No. 1 paper after 25 hr. development, seven spots of R_F values 0, 0.065, 0.17, 0.26, 0.39, 0.54, and 0.69 were obtained, and on Whatman No. 4 paper after 9 hr. development, seven spots of R_F values, 0, 0.10, 0.33, 0.46, 0.62, 0.72, and 0.86 were detected. It appeared, therefore, that at least

seven phosphorus-containing compounds were present. Table 3 shows that two of these corresponded in R_F values with α -glycerophosphate and inositol phosphate, respectively. The colour of the spot for inositol phosphate was lighter than that of the corresponding spot of the XSP factor. This may have been because in the latter case peptide material was present at the same position on the chromatograms.

Table 3. R_F values of α -glycerophosphate, inositol phosphate and phosphate fractions of *Klebsiella pneumoniae*

Solvent	Whatman Paper No.	Component or compound			
		α -Glycero-phosphate	Component of 'XSP' factor	Inositol phosphate	Component of 'XSP' factor
		R_F values			
<i>n</i> -Propanol + water + ammonia (sp.gr. 0.880); (60:10:30)	1	0.25	0.25	0.11	0.11
<i>iso</i> Propanol + water + ammonia (sp.gr. 0.880); (70:6:30)	1	0.54	0.54	0.39	0.39
	4	0.72	0.72	0.63	0.62

DISCUSSION

The results of this investigation show that pentose nucleic acid (PNA) cannot be estimated accurately in micro-organisms by the determination of either the optical absorption at 260 m μ , or the phosphorus content of the 'PNA fraction' of the Schmidt & Thannhauser (1945) procedure. This is due to the presence in this fraction of phosphorus-containing impurities and u.v.-absorbing impurities. An accurate estimation of PNA was obtained, however, by removing these impurities by paper electrophoresis and estimating the nucleotides spectrophotometrically. The use of this technique showed that both Gram-positive and Gram-negative organisms contained in their PNA fractions non-nucleotide, phosphorus-containing compounds (XSP factor). The results of Mitchell & Moyle (1950, 1954), from which it was concluded that only Gram-positive organisms contained this factor, were apparently due in part to their having ignored the presence of non-nucleotide u.v.-absorbing compounds.

The results obtained in this investigation for the XSP contents of the various micro-organisms were, where comparison was possible, much lower than those of Mitchell & Moyle when these authors' own method was used. It seems possible that this may have been due to the fact that in this work the organisms were grown in a Lemco + peptone medium at 37°, and that Mitchell & Moyle used a casein digest medium at 25°. In *Klebsiella pneumoniae*, an organism not analysed by Mitchell & Moyle, however, the XSP content was higher than any recorded by Mitchell & Moyle for a Gram-negative organism and higher than that found in this work in baker's yeast. When the present more accurate method of estimating PNA was used, however, the results for Gram-positive organisms were in fair agreement with those of Mitchell & Moyle but those for Gram-negative organisms were much higher.

The presence of 'XSP factor' in a Gram-negative organism has been confirmed both by the experiment with ^{32}P and by the isolation of non-nucleotide phosphorus-containing compounds from *Klebsiella pneumoniae*. Two of these have been tentatively identified as α -glycerophosphate and inositol phosphate, respectively.

The presence of non-nucleotide phosphorus-containing compounds in the PNA fraction of higher organisms has been reported by a number of workers. Davidson & Smellie (1952) found that this fraction from liver cells contained, in addition to the four nucleotides, at least six other phosphorus-containing compounds; and Logan, Mannell & Rossiter (1952) found that in nerve tissue, the non-nucleotide phosphorus-containing compounds accounted for more than 50 % of the total phosphorus of the PNA fraction. It appears, therefore, that non-nucleotide phosphorus-containing compounds occur in the PNA fraction of many organisms and are not peculiar to Gram-positive micro-organisms. There is therefore no evidence to connect the Gram-positive reaction of the micro-organisms examined here with the presence of an 'XSP factor', nor is there need to abandon the previous contention that the Gram staining properties are associated with a complex involving magnesium ribonucleate.

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EXPLANATION OF PLATE

- Fig. 1. Electrophoretogram of u.v.-absorbing components of PNA fraction of *Klebsiella pneumoniae*. $\times 0.4$.
- Fig. 2. Electrophoretogram of the PNA fraction of *Klebsiella pneumoniae*. ($\times 0.3$). X = autoradiograph showing components containing ^{32}P ; Y = photograph showing u.v.-absorbing components; A = adenylic acid; C = cytidylic acid; G = guanylic acid; U = uridylic acid; P_1 - P_{10} = unidentified phosphorus-containing compounds; origin line between P_1 and P_2 .

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Electrophoretogram of ultraviolet
absorbing components of
K. pneumoniae

Fig. 1

Fig. 2

A. S. JONES, S. B. H. RIZVI AND M. STACEY—ROLE OF PHOSPHORUS COMPOUNDS
IN GRAM REACTION. PLATE 1

(Facing p. 606)