THE ISOLATION AND COMPARISON OF PYROGENIC FACTORS FROM *PROTEUS VULGARIS*

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RECENT reviews on bacterial pyrogens have stressed their interest as therapeutic agents[1] and discussed their relationship to endotoxins[2].

Lipopolysaccharide may be derived both from the cells of a smooth strain of *Proteus vulgaris* and from the culture medium in which it has been grown for four days. For convenience and brevity we describe them as LPS(S) and LPS(E) respectively, the (S) referring to the lipopolysaccharides from the cell and (E) indicating the external source. Although workers using various organisms have studied the properties of the (S) type[3,4] and others the properties of the (E) type[5], the simultaneous isolation and comparison of the relationship between the two types of pyrogenic lipopolysaccharides from any organism does not appear to have been made, and in fact the lipopolysaccharide from *Proteus vulgaris* seems to have escaped detailed study.

Proteus vulgaris grows well in inorganic and organic fluid medium. When it had been established that the LPS(S) isolated by Westphal's process[6] from cells grown in inorganic medium was similar to that obtained from cells grown in nutrient broth, and that the latter yielded heavier growth of cells, the LPS(S) was more conveniently obtained in quantity from the cells grown in the organic medium. The yield of LPS(S) from the dried cells (5–10 per cent) contained 40–50 per cent of nucleic acid which could not be reduced below 2 per cent. (Table I.) As the loss during purification by this method is considerable five sedimentations were not exceeded in subsequent purifications.

TABLE I

REMOVAL OF NUCLEIC ACID FROM LPS(S) BY WASHING AND SEDIMENTATION AT 105,000 *g*

Sedimentation	1	2	3	4	5
Per cent residual Nucleic acid ..	15	7	5	2–3	2–3

In all instances the LPS(E) was obtained from the cell-free filtrate from the same organism, grown in inorganic medium for four days, by preliminary evaporation to low volume under reduced pressure followed by removal of inorganic salts by dialysis, extraction of bacterial protein with phenol, alcohol precipitation and repeated sedimentation in a high-speed centrifuge at values of up to 105,000 *g*, followed by freeze-drying.

The LPS(E) after concentration of the fluid medium and then dialysing was found to contain an antigenic protein-lipopolysaccharide complex and was completely free from nucleic acid which made purification of this

fraction easier. The protein LPS(E) complex could either be obtained as such by evaporation to suitable bulk and sedimentation in the high-speed centrifuge: or the protein could be removed by extraction with phenol as in the Westphal method for extraction from the dried cell.

Comparison of the two lipopolysaccharides showed that both contained a chloroform-soluble lipid, which could be split off on acid hydrolysis. Absorption spectra in the Dische test[7,8], showed a similar pattern: peaks at 408 mμ and 505 mμ indicated similar ratios of hexose to heptose in both compounds. Both fractions were shown by paper chromatography to contain hexosamine. On analysis the preliminary figures shown in Table II were obtained. Some further evidence of purity and homogeneity of the

TABLE II

COMPARISON OF LPS(S) AND LPS(E)

	C	H	N	P	Reducing sugars as glucose	Lipid	Minimum pyrogenic dose
LPS(S)	41·21	7·28	2·28	1·95	34 per cent	30–32 per cent	0·005 µg./kg.
LPS(E)	43·07	7·86	2·10	1·72	30 per cent	32–34 per cent	0·005 µg./kg.

two lipopolysaccharides was sought and both products were tested by the agar diffusion precipitin test[9], using the lipopolysaccharide as "antigen" against the serum of rabbits previously immunised against *Proteus vulgaris* cell suspension. The LPS(S) and LPS(E) in the prepurification stages gave two and several lines respectively. After sedimentation at 105,000 *g*, the LPS(S) gave only one zone while the LPS(E) gave a major coincident zone and a very faint zone suggesting an impurity. A mixture of the two lipopolysaccharides gave one major zone along with the very faint zone already mentioned. This was taken to indicate a close relationship but further detailed examination is in progress.

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DISCUSSION

The short communication was presented by MR. W. BOYLE.

THE CHAIRMAN. In what form was the phosphorus present in L.P.S.(S) and L.P.S.(E): from Table II there seemed to be little difference in their phosphorus contents. It seemed reasonable to suppose that the lipopolysaccharide required some portion of its molecule to be split off before part of it could pass through the bacterial cell into the culture medium. Might L.P.S.(S) be similarly split during its process of purification in view of the composition being so similar to that of L.P.S.(E)?

DR. J. G. DARE (Leeds). He had grown the *Proteus vulgaris* on surface cultures on solid media, washed them off carefully, to give a very concentrated suspension which, after being incubated, was filtered. When the filtrate was poured into another solvent, such as acetone, a precipitate of very highly active pyrogenic material was obtained in a yield corresponding to about 1 per cent in the original filtrate. By this method he avoided the lengthy and tedious concentration under reduced pressure. It was possible to obtain preparations which were active in rabbits in doses of less than 0·01μg./kg. He had tried a number of purification processes such as those recommended by Morgan, and he had always got back almost a quantitative yield (about 80 per cent) of the original material. He did not know whether that constituted purification or some loss in the process. Had the authors any reasons for thinking that his method might be unsatisfactory?

MR. G. A. STEWART (Dartford). It was stated that there was a hypothermic reaction with one preparation. Did they consider this to be a dual action of the pyrogen or due to some impurity associated with it?

MR. T. D. WHITTET (London). Had time-temperature curves for the two substances been compared?

DR. L. SAUNDERS (London). Might not these materials be break-down products from the cell wall which was being continually renewed? Had the ultra-violet absorption spectrum of the lipopolysaccharide been examined, and if so did it show unsaturated fatty acid in the active portion?

MR. H. D. C. RAPSON (Betchworth). Could an analogy be drawn between allergic reactions, such as from pollen extracts, and the pyrogen reaction?

MR. T. D. WHITTET (London). A large number of mucopolysaccharides had been extracted from allergic extracts recently. He had had some, and had not found any to be pyrogenic.

MR. BOYLE replied. The phosphorus figures quoted were for total phosphorus, and they had no evidence how it occurred. In the examination of the crude E material the nucleic acid absorption was at 260mμ, with much masking, probably due to the protein. After phenol extraction the polysaccharide material showed no peak at 260 mμ. It might be that in this extraction the nucleic acid had gone into the phenol layer and the polysaccharide into the aqueous layer. The surface culture method gave a great deal of extraneous matter, which they thought

would make purification more difficult. The material obtained from the cell-free filtrate had been used for some 10 years, and in that time very few toxic reactions had occurred in its use in rabbits. One of their aims was that eventually the materials would be used for clinical trials. They had been looking for an agent with as low toxicity as possible, and this method seemed to offer it. He agreed that the order of activity was not an indication of purity. After injection of these materials the first reaction was often hypothermic, and hyperthermia would occur later. Doses of about 10 μg. resulted in a temperature rise of 2-3° which might persists for 6 hours. Then it began to fall. If the same dose was again injected, there was a dramatic fall of temperature of about the same amount. They had found the curves to be almost exactly superimposable. With the E lipopolysaccharide the latent period was slightly shorter. When they gave higher doses, of about 1 μg., there was a double peak response, but curves were still superimposable. They had not examined the ultra-violet absorption of the lipopolysaccharide.

PROFESSOR TODD replied. To obtain the bacterial lipopolysaccharide by evaporating the medium was indeed a laborious method, but they had been anxious to find the substance which was present in injections. He agreed the indications were that the same substance was obtained by both methods. He thought that the presence of nucleic acid in one and its absence from the other was due to the location of the lipopolysaccharides in bacterial cell walls. The phenolic extraction disintegrated the cell and one got nucleic acid with it. There were two aspects to the fall in temperature. At the beginning of some injections there was a fall and then a continued rise, but with considerable overdosages there was a fall and no rise at all, the temperature remaining below the base line, presumably approaching the toxic level.