to those that occur in the course of hepatic injury such as carbon tetrachloride or ethionine poisoning (9–11). It was demonstrated that these toxicants produce fatty livers by interfering with hepatocellular lipoprotein synthesis or release (4). Furthermore, it was shown that the presence of numerous liposomes and the alterations in the Golgi apparatus, consisting of saccular dilatation and disappearance of their vacuolar content (also seen in the present experiments), are electron microscopic signs indicative of the failure of lipoprotein secretion (4, 6, 8, 12). Further investigations are, however, required to verify whether a similar mechanism is also responsible for the fatty degeneration caused by W-1372.

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* Fellow of the Medical Research Council of Canada.

Characteristics of a Highly Purified Pyrogenic Lipopolysaccharide

GAYLORD B. CASTOR, NATHAN KANTOR, EVERETT KNOLL, JOCELYN BLAKELY, JEAN K. NIELSEN, JOSEPH O. RANDOLPH, and AMIEL KIRSHBAUM

Abstract □ A highly purified lipopolysaccharide from *Klebsiella pneumoniae* was examined for qualitative chemical composition and, in a collaborative study, for pyrogenic activity. The principal constituents determined after hydrolysis were galactose and mannose, together with unidentified fatty acids. A dose of 0.001 mcg./kg. administered to groups of eight rabbits resulted in a high percentage of positive pyrogenic responses as defined by USP XVIII.

Keyphrases □ Lipopolysaccharide, pyrogens—isolation, purification, characterization, activity □ Pyrogenic lipopolysaccharide from *Klebsiella pneumoniae*—isolation, purification, and characterization, pyrogenic activity tested □ Bacterial pyrogens—isolation and purification of lipopolysaccharide from *Klebsiella pneumoniae*, determination of constituents and pyrogen activity

In a continuing effort to find a bacterial pyrogen that might be generally acceptable as a pyrogen control, scientists of the National Center for Antibiotics Analysis (NCAA) recently undertook to purify a previously known lipopolysaccharide from *Klebsiella pneumoniae* (ATCC 12833). Details of the methods used for earlier extracts of the organism were reviewed by Selzer (1).

In the latest study, a highly purified material was obtained. This paper reports on some qualitative chemical characteristics of the substance and presents the results of a collaborative study to determine its pyrogenic activity in rabbits.

EXPERIMENTAL

Isolation and Purification—The *K. pneumoniae* was maintained on slants containing 10 ml. of soybean–casein digest agar. For seeding purposes, a new slant was inoculated and incubated at 32–35° for 24 hr. With 3 ml. of sterile soybean–casein digest broth (USP XVIII), the growth was washed from the slant into a 38 × 200-mm. tube containing 100 ml. of the same broth. The broth tube was incubated at 32–35° for 24 hr. Approximately 4 ml. of the broth culture was transferred to each of 25 Roux bottles containing 300 ml. of soybean–casein digest agar with 0.25% dextrose. The bacterial suspension was spread over the surface with the aid of sterile glass beads. The Roux bottles were incubated at 32–35° for 3 days. The resulting growth was then washed from each Roux bottle with 20 ml. of sterile pyrogen-free distilled water. All washings were collected in a sterile, 1000-ml., screw-cap conical flask. One hundred milliliters of 37% formaldehyde was then added, and the mixture was allowed to stand overnight at room temperature. On the next day, the cells were centrifuged, washed with water to remove extracellular material, and freeze dried.

The basic purification procedure was that of Westphal and associates, as modified and described by Selzer (1).

Chemical Analysis—The lipid fraction of the purified material, analyzed by GC, appeared to contain several fatty acids, which cannot be identified at the present time. The carbohydrate moiety was hydrolyzed under various conditions, and the simple sugars were detected by descending paper chromatography.

The following solvent systems were used: I, 1-propanol–ethyl acetate–acetic acid–water (30:35:15:20); II, 1-propanol–ethyl acetate–pyridine–water (30:35:15:20); III, 1-propanol–ethyl acetate–pyridine–acetic acid–water (30:40:10:5:15); IV, 1-propanol–water (85:15); and V, 1-propanol–ethyl acetate–water (60:25:15) with filter paper[1].

The sprays for the neutral sugars were *p*-anisidine phosphate and aniline phosphate; for the amino sugar, ninhydrin and the Elson-Morgan reagent were used; and for 2-keto-3-deoxy octonate (2), thiobarbituric acid (3) was used.

The conditions of hydrolysis varied with the different sugars. Short periods of hydrolysis (5–15 min.) were used in attempts to detect dideoxyhexoses (4). Since the thiobarbituric acid spray is used to detect the dideoxyhexoses as well as 2-keto-3-deoxy-octonate, these chromatograms were checked for both substances.

To detect the neutral and amino sugars, the lipopolysaccharide was refluxed with 0.4 *N* sulfuric acid for 1 hr. The solid lipid material that appeared on cooling was extracted with hexane, evaporated under nitrogen, and analyzed by GC. The aqueous solution was hydrolyzed for 4 hr. more, yielding a brown solution, which was clarified with Darco S51, neutralized with $BaCO_3$, filtered, and freeze dried. The dried material was dissolved in a minimum of water, and several microliters were spotted on the paper for chromatography.

The amino sugars were more readily ascertained by means of HCl hydrolysis. The lipopolysaccharide was refluxed with 0.4 *N* HCl for 1 hr., the lipid was removed, and the hydrolysis was continued for 5 hr. longer. The brown solution was clarified with Darco S51, neutralized with Dowex-3, and freeze dried. The dried material was treated as described previously for chromatography.

Heptose (usually glycero-manno-heptose), present in many lipopolysaccharides, was checked colorimetrically by the Osborn modification (5) of the cysteine–H_2SO_4 reaction.

The purified pyrogen was stored in a desiccator. Prior to testing for pyrogenic activity, a determination of loss on drying in the vacuum oven was made. Uptake of moisture on exposure was also determined.

Molecular weight was determined by ultracentrifugation. An elemental analysis was also made.

Pyrogenic Activity[2]—Each participating laboratory was furnished a sample of the preparation and a uniform protocol for testing. The sample was to be vacuum dried, cooled under desiccation, and weighed. A solution containing 100 mcg. of sample/ml. of sterile pyrogen-free water was prepared first. An aliquot was then diluted to 1.0 mcg./ml. with the same diluent. An aliquot from the second solution was further diluted to 0.01 mcg./ml.; from this last solution, the test solutions listed in Table I were prepared. The basic test procedure was that described in USP XVII (p. 863)[3], except that 32 rabbits were to be injected at the same hour, 8 for each dose level, with 1.0 ml./kg. administered by rapid intravenous injection. Upon completion of the test, each laboratory was requested to send their results to NCAA for summarization and analysis.

RESULTS

Chemical Analyses—Short-period hydrolysis of the polysaccharide fraction failed to expose any dideoxyhexoses when chromatograms of the hydrolysate were sprayed with thiobarbituric acid. 2-Keto-3-deoxyoctonate, however, was detected on all of these chromatograms.

After H_2SO_4 hydrolysis, the principal constituent was found to be galactose, followed closely by mannose. Traces of some amino sugars were also present.

[1] Whatman No. 1, Whatman No. 2, and S & S 589 Blue Ribbon.

[2] The following 12 laboratories currently engaged in pyrogen testing participated in the collaborative study: Abbott Laboratories, N. Chicago, Ill.; Belgian Pharmaceutical Association, Brussels, Belgium; Bristol Laboratories, Syracuse, N. Y.; Commonwealth Serum Laboratories, Parkville, Victoria, Australia; Connaught Medical Research Laboratories, Willowdale, Ontario, Canada; Lederle Laboratories, Pearl River, N. Y.; Eli Lilly and Co., Indianapolis, Ind.; Merck Sharp & Dohme, West Point, Pa.; National Center for Antibiotics Analysis, Food and Drug Administration, Washington, D. C.; Chas. Pfizer and Co., Inc., Brooklyn, N. Y.; E. R. Squibb and Sons, Inc., New Brunswick, N. J.; and Wyeth Laboratories, Inc., West Chester, Pa.

[3] USP XVIII is equally applicable.

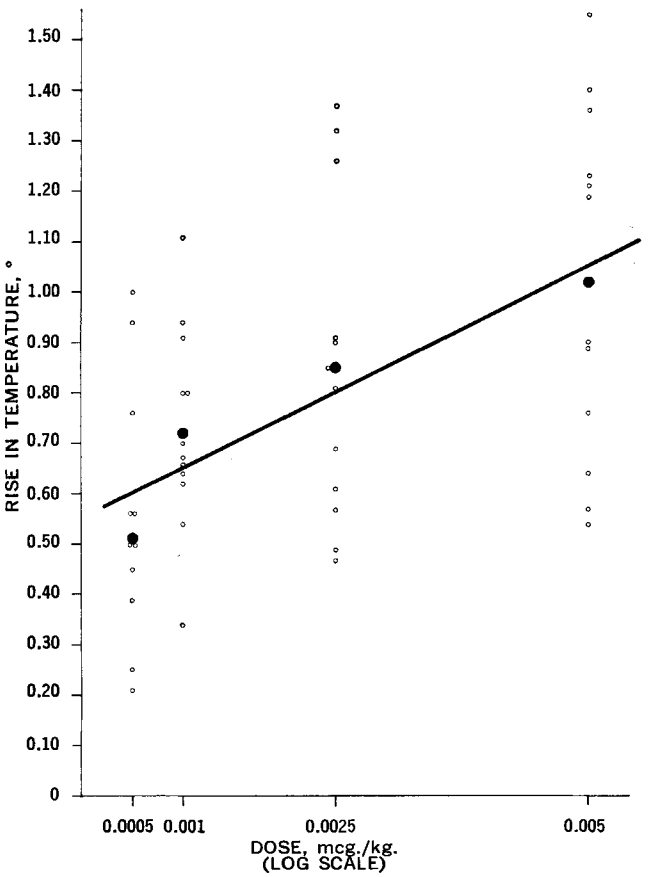

Figure 1—*Distribution of the average temperature rise obtained by each laboratory at each dose level. Key: ● = average for each dose.*

After HCl hydrolysis, galactose and mannose were, again in that order, the major constituents. Glucosamine was also detected.

Heptose was not detected colorimetrically. Paper chromatography was not used inasmuch as the large amount of galactose present would have interfered with the determination.

In summary, the carbohydrate components of the lipopolysaccharide appear to be galactose, mannose, 2-keto-3-deoxy-octonate, and glucosamine, in descending order of magnitude.

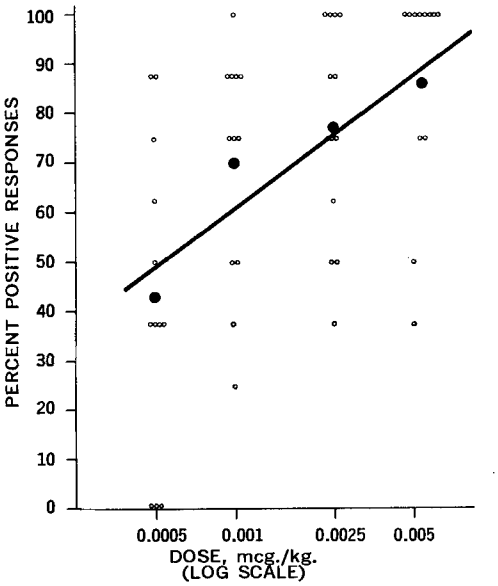

Figure 2—*Distribution of the percent of rabbits showing pyrogenic rises for each laboratory at each dose level. Key: ●, average for each dose.*

Table I—Results of Collaborative Assay[a] of NCAA Pyrogen, 1969

Laboratory	Test Doses: 0.0005 mcg./kg. Number of Rises	Sum	Average Rise	0.001 mcg./kg. Number of Rises	Sum	Average Rise	0.0025 mcg./kg. Number of Rises	Sum	Average Rise	0.005 mcg./kg. Number of Rises	Sum	Average Rise
A	5/8	4.5°	0.56°	7/8	5.6°	0.70°	8/8	7.4°	0.91°	8/8	11.2°	1.40°
B	6/8	6.1°	0.76°	7/8	7.5°	0.94°	7/8	10.1°	1.26°	8/8	12.4°	1.55°
C	3/8	3.1°	0.39°	6/8	5.3°	0.66°	6/8	4.9°	0.61°	6/8	5.1°	0.64°
D	3/8	4.00°	0.50°	4/8	5.35°	0.67°	5/8	6.50°	0.81°	8/8	9.95°	1.23°
E	0/8	1.65°	0.21°	4/8	5.00°	0.62°	3/8	3.75°	0.47°	4/8	4.30°	0.54°
F	7/8	7.5°	0.94°	7/8	7.3°	0.91°	8/8	11.0°	1.37°	8/8	9.5°	1.19°
G	3/8	3.6°	0.45°	7/8	6.4°	0.80°	7/7	6.3° (7.2°)	0.90°	7/7	6.2° (7.1°)	0.89°
H	4/8	4.5°	0.56°	6/8	6.4°	0.80°	7/8	6.8°	0.85°	8/8	9.7°	1.21°
J	7/8	8.1°	1.00°	8/8	9.0°	1.11°	8/8	10.6°	1.32°	8/8	10.9°	1.36°
K	3/8	4.0°	0.50°	6/8	5.1°	0.64°	6/8	5.5°	0.69°	8/8	7.2°	0.90°
L	0/8	2.0°	0.25°	2/8	2.7°	0.34°	4/8	4.6°	0.57°	6/8	6.1°	0.76°
M	0/8	−1.0°	−0.12°	3/8	4.3°	0.54°	4/8	3.9°	0.49°	3/8	4.6°	0.57°
Average		4.08°	0.51°		5.82°	0.72°		6.85°	0.85°		8.17°	1.02°
Number of pyrogenic rises		41/96			67/96			73/95			82/95	
Percent		43			70			77			86	
Range of temperature changes		−0.8–+1.6°			−0.1–+1.6°			−0.4–+2.0°			−0.3–+2.2°	

[a] *Departures from the protocol:* Laboratory A used three unrestrained rabbits at 0.0025 mcg./kg. and eight at 0.005 mcg./kg. Manual rectal thermometers were used as well as electronic sensors. Laboratory B did two complete determinations. The one tabulated utilized two weighings of the pyrogen for preparation of the dilutions. Laboratory G, for lack of space, used only seven rabbits for the two higher doses. The figures in parentheses are extrapolations of the sums to eight rabbits. In two of Laboratory K's test groups, the control temperature variation between the rabbits in each group was considerably more than the 1° permitted by the USP.

In the vacuum oven, the purified pyrogen lost approximately 2.9% moisture. On exposure, it proved moderately hygroscopic, stabilizing as the moisture content reached almost 12%.

The molecular weight was 4.77×10^6. The elemental analysis was as follows: C, 45.45%; H, 6.13%; N, 1.15%; and P, 0.065%.

Collaborative Pyrogenic Evaluation—A summary of the findings of each laboratory is given in Table I. Although response to the pyrogen varied considerably, as recorded by the different laboratories, the overall average temperature rise increased with increasing dosage. Likewise, the number and percent of rabbits showing pyrogenic rises increased as the dosage increased. (According to USP criteria, a pyrogenic rise is an elevation of 0.6° above the initial, or control, temperature reading.)

The latitude and correlation of the temperature rises, averaged for each laboratory, are better visualized in the distribution graph (Fig. 1). The upward bias at the successively higher dose levels is readily apparent. The slope of the regression line is $0.01 \pm SE = 0.0025$, with the intercept at 0.55°. Thus, a positive slope is indicated, which is significantly different from zero at a probability level of $p < 0.001$.

Figure 2 is similarly constructed to show the distribution of the percent of rabbits giving positive responses for each laboratory at each dose. The slope of the regression line of the mean percentages is 28% per log dose (base 5). It is significant at $p < 0.001$.

DISCUSSION

A quantitative analysis of the chemical components of the *Klebsiella* pyrogen was not made. The estimated qualitative composition, however, agrees well with that given by Berger *et al.* (6) in a summary of determinations by investigators for other known pyrogens. The large percentage of galactose present is intriguing in view of the abundance of glucose found in polysaccharides in general.

The wide variation in results obtained by the 12 laboratories in the collaborative assay emphasizes the reasons preventing general adoption of a pyrogen standard. In Table I, for example, Laboratory J and Laboratory M represent extremes of responsiveness as measured both by the number of rabbits having pyrogenic rises and by the average rise at each dose level; J displayed the greatest sensitivity, M the least, and the spread is considerable.

One aim of the study was to establish the test dose that reasonably could be expected to produce a minimal pyrogenic response as defined by the USP. This dose is 0.001 mcg./kg.; 10 of the 12 laboratories reported a sufficient number of rabbits responding (four or more out of eight), and 11 of the 12 reported a sufficiently high sum of the temperature rises (more than 3.7°).

The highest dose used (0.005 mcg./kg.) was expected, on the basis of preliminary trials, to give a very high percentage of individual positive responses. Eight laboratories did, in fact, report 100% response, yet the total pyrogenic rises occurred in only 86% of the rabbits injected. To test the sensitivity of individual animals, a higher dose of this material might have to be used.

Other characteristics of this highly potent material are presently under study.

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