Isolation from *Penicillium gilmanii* of a Substance that Causes Leucocytosis in Rabbits.* (31438)

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Bacteria(1), molds(2) and inflammatory exudates(3) are well known as sources of substances that upon injection into animals produce an increase in the animal's body temperature. This increase in temperature is usually associated with an initial leucopenia followed later by a leucocytosis(4,5). The biologically active material from bacteria has been traced to a lipopolysaccharide(6). It was considered to be of interest to attempt to isolate and characterize the biologically active material from a mold for comparison with that obtained from bacteria.

Methods. Preliminary experiments showed that when *P. gilmanii* was grown for approximately one month as a surface culture on a Czapek-Dox medium, intravenous injection into rabbits of the equivalent of 0.01 ml of the metabolic solution produced a several degree increase in the animal's body temperature as well as a marked leucocytosis. *P. gilmanii* was therefore chosen as the mold from which to attempt the isolation of the substance or substances responsible for causing the increased temperature and in particular the leucocytosis.

Rabbits (New Zealand whites, 2 to 3.5 kg in weight) were used for bioassay. In establishing the method of purification some of the animals were reused at 2-week intervals. The activity of the final product was determined in a group of 45 virgin rabbits at 10-fold steps in concentrations of from 0.001 μg to 10 μg per kg.

Rectal temperatures were taken on a telethermometer.† Blood cells were counted by standard methods with a hemacytometer(7) or a Coulter counter. Wright's stain was used for staining the cells for differential counts(7).

Injections were made intravenously in one ear and, for total and differential counts, samples of blood were taken at hourly intervals from the other ear.

Fractions in aqueous solution, to be used for bioassay, were freeze dried. Fractions in organic solvents were evaporated to dryness under reduced pressure at room temperature. Approximately a milligram of the fraction was dissolved in 10 ml of pyrogen-free isotonic saline,‡ and filtered through a Millipore® filter.§ Appropriate dilutions were then made in isotonic saline.‡

All glassware was cleaned by heating at 100° in a 1:1 mixture of *conc* sulfuric and *conc* nitric acids. The glassware was then thoroughly washed with tap water, followed by distilled water and rinsed with acetone. Serum bottles to be used as containers for samples for bioassay were, in addition, autoclaved at 15 psi and pipettes were sterilized at 160-70°. Disposable syringes¶ were used for injections. All solvents were reagent grade and were redistilled before use.

It was early observed that continuous extraction of the culture medium with initially anhydrous reagent grade diethyl ether for 6 to 8 hours separated the material that caused a temperature increase from that which caused a leucocytosis. The material causing a temperature increase was extracted into the ether‖ while the leucocytosis causing material remained in the aqueous phase.

The leucocytosis causing material was dialysable through cellophane and could be removed from an acidified solution by Amberlite 45A (in the basic form).** It could be

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† YSI-Model 43, Yellow Springs Instrument Co., Inc., Yellow Springs, Ohio.

‡ Normal Saline N-S-S. (Pyrogen Free) prepared by Cutter Laboratories, Berkeley, Calif.

§ Manufactured by: Millipore Filter Corp., Bedford, Mass. A 10 ml syringe with Swinny adaptor was used.

¶ Tomac disposable 1 ml tuberculin type syringe.

‖ The isolation and characterization of the temperature causing material will be reported later.

** I should like to express my appreciation to the Rohm and Haas Co., Philadelphia, Pa. for supplying

eluted from the Amberlite by dilute aqueous ammonia.

The following procedure for isolation of the leucocytosis causing factor was devised on the basis that discarded fractions caused no leucocytosis when amounts approximately equivalent to the amount found in 0.01 ml, 0.1 ml, 1 ml and 10 ml of the culture fluid were injected into rabbits.

Isolation procedure. Two liters of culture fluid from *P. gilmanii,* grown as a surface culture on a Czapek-Dox medium for from 4 to 6 weeks (pH 4.5 to 5.2) was filtered through fluted filter paper and then continuously extracted with diethyl ether for from 6 to 8 hours. The extracted culture medium was then poured through 50 g of Amberlite 120 (H) contained in a fluted filter. The filtrate (pH 2.5 to 3.1) was immediately filtered through a column of Amberlite 45A (OH) (600 g in a column 5 × 54 cm). When the culture medium reached the top of the column, distilled water was added and the column was washed free of adsorbed glucose with additional distilled water (at least 14 liters). The biologically active material was then eluted from the column by adding at the top of the column 30 ml of *conc* ammonia water diluted to 250 ml with distilled water. Additional distilled water was added as the level approached the top of the resin column. After approximately 500 ml had passed through the column the eluate was slightly alkaline to test paper and 1200 ml was then collected. The 1200 ml was evaporated to 40 to 50 ml on a rotary evaporator under reduced pressure at 40°-50° (bath temperature) and freeze dried. The dried solid was extracted several times with boiling methanol (5 × 150 ml) and filtered. The combined filtrates were treated with 5 g of $Pb(OAc)_2 \cdot 3H_2O$ (dissolved in 50 ml of methanol) and filtered through a fritted glass filter. The insoluble portion was washed with 100 ml of methanol. The filtrate was treated with hydrogen sulfide gas, filtered through fluted filter paper and the insoluble lead sulfide washed with 75 ml of methanol. The methanol solution was evaporated to dryness at 40°-50° (bath temperature) under reduced pressure. The product, a thick sirup containing considerable crystalline material, was extracted with *normal* propyl alcohol (10 ml) and filtered. The insoluble portion was washed 3 times with 10 ml portions of *normal* propyl alcohol and filtered. The combined filtrates were diluted 1:1 with methanol and passed through 20 g of Amberlyst-15 (acid form, equilibrated with methanol) in a 2 cm × 60 cm column. The column was washed with 600 ml of methanol. The effluent and washings from the column were collected in a flask containing 2 g of lead carbonate and the mixture was immediately evaporated to dryness at 40°-50° under reduced pressure. The residue was extracted with dioxane (3 × 50 ml) and filtered. The filtrates were combined, treated with hydrogen sulfide gas, filtered and evaporated to dryness at room temperature under reduced pressure. The resulting sirup was extracted with *anhydrous* diethyl ether (3 × 25 ml), filtered, and the ether solution forced through 20 g of silicic acid†† in a 2 cm × 50 cm column under 20 cm Hg pressure. The column was washed with 75 ml of *anhydrous* diethyl ether followed by 75 ml of ethyl acetate. The active material was then eluted with benzene containing 5 ml of methanol per 100 ml. On the addition of the benzene-methanol mixture to the top of the column a translucent zone appeared. When this zone had just washed off the column, 200 ml of the benzene-methanol mixture was collected, filtered through a (medium) fritted glass filter and evaporated to dryness at room temperature under reduced pressure. The product, a sirup, (yield 5 to 20 mg) could not be obtained crystalline. Thin layer chromatography on silica gel,‡‡ however, showed a single zone when the slides were developed with 5 and 10% methanol in benzene, or dioxane, or various concentrations of dioxane in ether. Ultraviolet light, or 2,4-

the Amberlites and Amberlyst gratis. The resins were cycled several times and washed with 20 to 30 liters of distilled water before use.

†† Silicic acid (Reagent grade) powder.

‡‡Silica gel 80.76, obtained from Research Specialties Co., Richmond, Calif. To this was added zinc silicate (250 mg per 30 g) to produce a fluorescent adsorbent.

dinitrophenylhydrazine in 2 *N* HCl (as a spray) or iodine vapor was used to make the zone visible. The zone was biologically active when assayed within a few hours. It lost activity slowly, however, on standing at room temperature for a period of days and in a matter of hours at 50°. Concurrent with the loss in activity more and more of the material became insoluble in diethyl ether. The ether insoluble material had no biological activity. When the sirup was neutralized with calcium, sodium, ammonium or potassium hydroxides to form the corresponding salt, the solutions were stable for several months. The salts were isolated by quickly titrating an aqueous solution to pH 7 with the appropriate hydroxide, concentrating the solution to dryness at room temperature under reduced pressure and precipitating the salt from an ethanol solution by the addition of dioxane. The sodium salt was very hygroscopic. The salts were equivalent in biological activity. The calcium salt was the simplest to prepare. Ten to 15 mg of solid calcium hydroxide was added to approximately 40 mg of the sirup dissolved in 1 ml of water. *Absolute* ethanol (20 ml) was added and the solution filtered through a fritted glass filter and evaporated to dryness under reduced pressure at room temperature. The salt could be further purified by precipitating it from aqueous solution by addition of dioxane or from a methanol solution by addition of *normal* propyl alcohol.

Elemental analysis on the sirup, the *bis*-2,4-dinitrophenylhydrazone, the calcium salt, and the heptabenzoate, together with a molecular weight by thermal diffusion, indicates the molecular formula of the leucocytosis causing factor to be $C_{18}H_{29\text{-}31}NO_9$. Titration of the sirup indicated one acidic group. Infrared spectra and spot test analysis(8) indicate that the acidity is due to an enol group. It is suggested therefore that the compound be named *Leucogenenol.*

Discussion. No correlation could be found between quantity of *Leucogenenol* injected and degree of leucocytosis. At the level of 0.001 μg per kg 2 of 6 animals responded at the end of 3 to 6 hours with counts approximately 5 times their normal. Four of the animals, however, showed only a one and a quarter to two-fold increase. The animals returned to normal after 24 hours. All animals showed a differential increase in neutrophils. At the 0.02 to 0.2 μg per kg all animals showed a 2- to 3-fold increase in white cell count. The maximum occurred at unpredictable intervals of from 4 to 12 hours. The total count, however, remained elevated for a number of days. As a rule, at the microgram per kilo level, no marked leucocytosis was observed for 12 to 20 hours. At the end of this time, however, the total count was elevated (1½- to 2-fold) and remained elevated for approximately a week. A few observations suggest that at the microgram or more level of injection there is a marked transient leucocytosis within the first hour.

Up to a milligram of the calcium salt could be injected into a mouse without any toxic reaction. Relatively large quantities (4 μg per mouse) produced only a slight leucocytosis followed later by a leucopenia. After 12 to 24 hours the counts slowly returned to normal. The lymphocyte count was only slightly affected. Thymectomized mice showed a similar picture.¶¶

Summary. A compound, chromatographically homogeneous, was isolated from the metabolic products of *P. gilmanii.* This compound when injected into rabbits in quantities of from 0.001 μg to 10 μg per kg causes a leucocytosis without a concurrent increase in body temperature. The material is not toxic to mice when injected in milligram quantities. Preliminary analysis suggests a minimum molecular formula of $C_{18}H_{29\text{-}31}NO_9$. The compound is apparently an enol, hence the name *Leucogenenol* is suggested for the compound.

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¶¶ To be published later.

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Depression of Plasma FFA Levels in Unanesthetized Dogs by Single Intravenous Doses of Prostaglandin E_1. (31439)

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Prostaglandin, an acidic lipid fraction with vasodepressor and smooth muscle-stimulating properties, was first reported by Goldblatt(1, 2) and by von Euler(3,4). In recent years studies by Bergström and collaborators have led to the isolation and full characterization of a large family of structurally related prostaglandins(5-7). Members of the family have been shown to decrease basal rates of glycerol release and to inhibit the fat-mobilizing effects of lipolytic hormones in rat and human adipose tissue *in vitro*(8-10). Prostaglandin E_1 (PGE_1) was shown to block the *in vivo* fat-mobilizing effect of epinephrine in the anesthetized dog with little or no inhibition of its glucose-mobilizing effect(9,11). Bergström *et al* have shown in unanesthetized man an unexpected *rise* in plasma FFA during continuous infusion of small doses of PGE_1 alone and only a very limited inhibition of the fat-mobilizing action of simultaneously infused epinephrine(12).

Work reported here shows that intravenously administered PGE_1 counteracted the lipid-mobilizing effect of intravenous epinephrine in unanesthetized dogs. Glucose responses were little affected. Injection of large single doses of PGE_1 alone consistently produced a pronounced drop in basal plasma FFA levels which could not be explained as a secondary effect of the accompanying vasodepression.

Methods. Mongrel male dogs fasted overnight were trained to stand for several hours supported by a cloth sling. A venous catheter was placed in each foreleg: one for test substance injection, the other for blood sampling. Blood samples were heparinized and immediately chilled. Analyses were begun no more than 3 hours after sampling. Plasma FFA analyses were done according to the method of Dole(13) except that isooctane was used in place of heptane. Glucose determinations were performed enzymatically using glucose oxidase ("Glucostat" reagent sets, Worthington Biochemical Corp., Freehold, N. J.). PGE_1, kindly provided by Dr. Sune Bergström, and PGE_1-217 (9-keto-15-hydroxy-prosta-10,13-dienoic acid), a gift of Dr. J. E. Pike, Upjohn Co., were prepared in aqueous solutions containing 0.1 ml of ethanol for each mg of PGE_1 and diluted with 0.85% NaCl as necessary. The total amount of ethanol injected was never more than 0.1 ml. Stock epinephrine solutions (1 mg base/ml) were prepared from the bitartrate and diluted in 0.85% NaCl for use.

Hemodynamic measurements were made on trained conscious dogs. Externalized carotid artery-jugular vein shunts had been placed in these dogs at least 2 days prior to study. Blood pressure measurements were made from the arterial side of the shunt using a Statham strain gauge and Sanborn recorder system. Injections and sample withdrawals were made