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Qualitative Detection of Muramic Acid in Normal Mammalian Tissues

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Rat tissues were extracted with 8% trichloroacetic acid, and the products were hydrolyzed with hydrochloric acid and purified. Fluorescamine derivatives were made and subjected to thin-layer chromatography; material with an R_f corresponding to the authentic muramyl derivative was obtained. It was oxidized with periodate, and the resulting formaldehyde was identified fluorimetrically. Alternatively, treatment with base released D-lactate (β-elimination), which was identified fluorimetrically by reduction of NAD to NADH with D-lactate dehydrogenase. The data indicate that small muramyl compounds, presumably peptides of bacterial origin, are normally present in rat liver, brain, and kidney. The functions of muramyl compounds are suggested by much recent work.

Muramyl peptides have been shown to act as modulators of some physiological activities in mammalian tissues. These substances, which are fragments of bacterial cell walls, possess, for example, activity as immunoadjuvants (14), direct primers of some macrophage functions (7, 18, 27), pyrogens (14), antitumor agents (3), and inducers of contractility of smooth muscle (17). Some have also been reported to be somnogens (9–11), i.e., promoters of slow-wave sleep. The release of peptidoglycans by macrophages from ingested bacterial cell walls (M. W. Vermeulen and G. R. Gray, Fed. Proc. **42**:1221, 1983) has been described recently. Studies of the clearance or degradation of these compounds in mice in vivo and in vitro have been carried out (12, 20, 24, 29).

Because of the important functions of muramyl peptides, as outlined above, it was of interest to examine normal mammalian tissues to determine whether muramic acid-containing substances are indeed present. This report describes the detection, on a microscale, of small acid-soluble muramyl entities, presumably peptides, in rat tissues as muramic acid per se. In particular, we concentrated on the establishment of specificity to be sure that the substance actually detected was indeed muramic acid.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats were used. The average body weight was 200 to 250 g.

Chemicals and reagents. All chemicals were analytical grade; muramic acid, fluorescamine, *N*-acetylneuraminic acid (NANA), D-glucosamine, D-galactosamine, and D-lactic dehydrogenase were obtained from Sigma Chemical Co. Radioactive muramic acid, labeled on carbon-1 of the lactate moiety, was from Research Products International Corp. Cation-exchange resins were from Bio-Rad Laboratories. The chromatoplates were silica gel plates (20 by 20 cm) from Fisher Scientific Co.

Instruments. The spectrophotofluorometer was an Aminco-Bowman Instrument 268F. The Speed Vac concentrator was from Savant Instruments Inc.; the long-wave UV lamp used was from Ultraviolet Products, Inc. An International refrigerated centrifuge model PR-2 was used. Counting of radioactivity was done on a Tracor Delta 300 liquid scintillation system.

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Sterile conditions. To obtain conditions as near to sterility as possible, all the glassware and surgical instruments were boiled for 1 h, soaked in 75% alcohol for 1 h, and then dried in an oven at 100°C. The deionized water was boiled a second time, and the ion-exchange columns were freshly prepared and kept at 0°C. The carotid arteries of the animals were cut, and the blood was removed under sterile conditions. The whole brain, kidney, and liver were removed and placed on ice as quickly as possible.

Extraction and hydrolysis of muramyl compounds and purification of muramic acid. An outline of the procedures for extraction, hydrolysis of extracted material, and purification of muramic acid is shown in Fig. 1.

The tissues were cut into many slices and homogenized with a motor-driven glass homogenizer (Potter-Elvehjem) in a fivefold volume of 8% trichloracetic acid (TCA) at 0°C. The time from the start of killing the animals to completion of homogenization was 10 min. After homogenization, the tubes of homogenate were placed on ice with occasional shaking for 20 min; they were then centrifuged at 15,000 × g for 20 min. The precipitate was reextracted twice. The supernatant fluids were combined, and the TCA was extracted with 10 ml of ether; usually, five extractions sufficed. The final aqueous phase was dried with a Speed Vac concentrator. The dried samples were promptly hydrolyzed with 1 ml of 6N HCl in sealed test tubes at 100°C (boiling water) for 6 h after air was removed with nitrogen (10).

The residue after vacuum drying was extracted three times with 0.5 ml of methanol each time. Centrifugation at 1.5×10^4 rpm for 10 min between extractions and after the third extraction was used to separate the extract from the solids. The combined methanolic extract was dried under low pressure on the Speed-Vac concentrator.

For purification, cation-exchange chromatographic columns (AG50W-X8; 0.6 by 6 cm, 200 to 400 mesh, 5.1 meq/g [dry weight], 1.7 meq/ml of resin bed) were prepared as described previously (28) and as modified in minor details by M. Schwarzschild and M. L. Karnovsky (unpublished data). The columns were washed with 10 ml of boiled, deionized water and then equilibrated with 0.1 N HCl to complete the conversion to the hydrogen form.

To separate muramic acid and amino sugars from neutral sugars and other neutral compounds, the samples were dissolved in 1 ml of 0.1 N HCl and passed through columns in the hydrogen form. The columns were then washed with 8

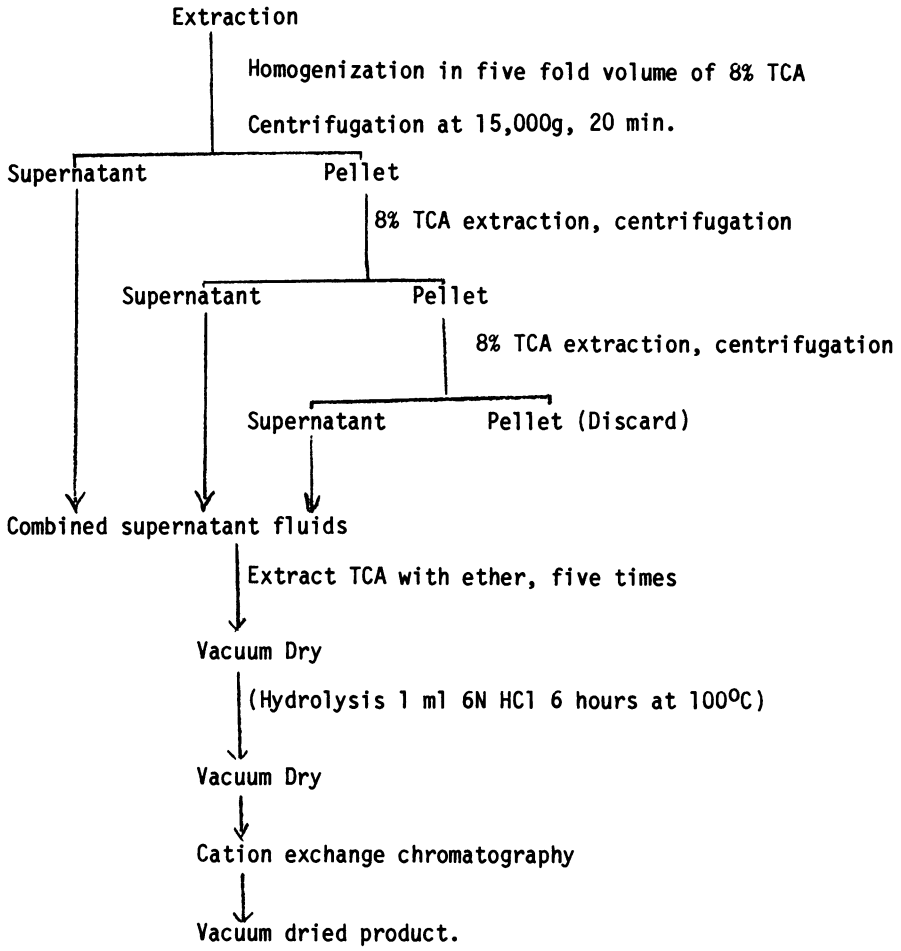

FIG. 1. Scheme for the extraction and purification of muramic acid from brain, liver, and kidney tissue.

ml of 0.1 N HCl. Amino sugars and muramic acid were then eluted with 8 ml of 0.5 N HCl, and the solution was evaporated on the Speed Vac concentrator. Muramic acid was separated from glucosamine with a similar cation-exchange chromatographic column. Before use, it was washed with deionized water and then rinsed with 8 ml of 1 N potassium hydroxide and equilibrated with 0.15 M potassium acetate buffer (pH 5.6). The sample was passed through the column in the potassium form. Muramic acid was eluted with 8 ml of the same buffer, and the solution was dried as described above. To remove the potassium salts, the dried samples were dissolved in 0.5 ml of 0.1 N HCl, and applied to a third column in the hydrogen form. The muramic acid was eluted with 6 ml of 0.5 N HCl. After drying, the samples were ready for the thin-layer chromatography (TLC) step.

Identification of muramic acid. Muramic acid identification was performed on the fluorescamine derivative (fluorophor) as described for amino acids and peptides (6, 26) by TLC on silica gel.

Preparation of fluorophors. Standard muramic acid and the purified hydrolyzed extracts of brain, liver, and kidney were reacted with fluorescamine, as described below. Samples containing ca. 200 pmol of muramic acid dissolved in 10 μl of 0.1 M sodium borate (pH 9.0) and 10 μl of fluorescamine in dioxane (20 mg/100 ml of dioxane) were added to a tube, which was held on a Vortex mixer. Several control or reference samples were prepared. For example, NANA was hydrolyzed with 1 ml of 6 N HCl at 100°C for 6 h in a sealed tube, and the fluorophor was made as described above. NANA, glucosamine, or galactosamine was also treated with the fluorescamine reagent.

The use of known compounds helped to define the location of known muramate of the tissue extracts in the TLC procedure described below. As a control for muramate that might have been introduced adventitiously during the procedures, the entire process outlined above was carried out with omission of the tissue sample.

After the fluorescamine reaction, a portion of the contents of each tube was applied to the chromatoplates (silica gel plates, 20 by 20 cm). Samples (standards and controls) were spotted in 3-μl amounts. After drying, the chromatogram was developed in the ascending mode in a dark environment for 3 to 4 h. The following solvent systems were selected: solvent system A, *n*-butanol–isopropanol–water (1:2:1); solvent system B, *n*-butanol–ethanol–water (8:8:5). After drying in the ventilation hood, the plates were sprayed with 5% triethanolamine to stabilize the spots (6). Fluorescent spots could be observed on the chromatograms under long-wave UV light. The sensitivity for each spot was about 10 pmol. About five to six spots were seen with the liver extract, but brain and kidney extracts gave less complex patterns (three to four spots).

Confirmation of identity of muramyl spots. The muramyl derivative recognized by migration analogous to that of the

standard muramyl fluorescamine derivative was confirmed by TLC in a second dimension and by two additional methods.

(i) Periodate oxidation of the putative muramic acid derivative. Periodate oxidation was employed to produce formaldehyde from the primary carbinol of muramate. This was condensed with acetyl acetone and ammonia to form a fluorescent 3,5-diacetyl-1,4-dihydrolutidine (5, 16). Silica gel samples (2.0 to 4.0 mg), i.e., the relevant fluorescent spots mentioned above, were scraped from the plates developed in one dimension. After authentic muramyl fluorophore was developed, spots were also taken as a control. The same weight of silica gel scraped from the same plate in a position without fluorescence served as an additional control. Periodate oxidation was carried out under basic conditions (22). To the silica gel was added 250 μl of 1 N sodium bicarbonate (pH 10), and the mixture was agitated for 10 min with a Vortex mixer. The oxidation was started by the addition of 250 μl of 0.04 N periodic acid and allowed to proceed at room temperature with occasional shaking for 1 h. The mixture was then centrifuged at 15,000 rpm for 15 min. Supernatant (450 μl) was collected. Sodium arsenite (50 μl, 1.2 N) and HCl (50 μl, 3 N) were added with gentle shaking until the yellow color disappeared. Freshly prepared reagent (500 μl of 2 M ammonium acetate–0.02 M acetyl acetone in water) was added, and the fluorescence was developed at 37°C for 1 h (5, 16). The fluorescence spectrum was then determined.

(ii) β-Elimination of D-lactic acid from the putative muramic acid derivative. Base-catalyzed β-elimination of D-lactic acid from muramic acid and its derivatives was employed (8, 23). The lactic acid was converted to pyruvate and NAD was converted to NADH by use of D-lactate dehydrogenase. We took 0.5 to 1.0 mg of silica gel with the fluorophor, as mentioned above, from the chromatogram. Standard muramyl spots (ca. 50 pmol) and blank spots were also examined. To the silica gel was added 1 ml of 4 N NH_4OH at 37°C for 16 h with occasional shaking. The tubes were then centrifuged at 15,000 × *g* for 20 min, and the supernatant was dried with a Speed Vac concentrator. After drying, 0.5 ml of 0.4 M hydrazine buffer (pH 8.5), 0.05 ml of NAD (5 nmol), and 37.5 U of D-lactic dehydrogenase were added (cf. reference 15). The reaction was allowed to proceed at 25°C for 30 min. Sodium hydroxide (2 N) was added to pH 12.3 at 60°C. Five minutes were allowed for destruction of the excess NAD (15). The fluorescence spectrum of NADH and relative fluorescence intensity were determined and compared with the standard control and blank controls.

RESULTS

Our approach involved the extraction of the tissue and the hydrolysis of muramic acid-containing components as soon as possible. By this strategem, we hoped to avoid the appearance of muramate as a result of potential bacterial contamination of the solutions after isolation from the tissue. Such contamination would not likely be as free muramic acid. In preliminary experiments, we used traces of ^{14}C-labeled muramate to follow that substance through the multiple steps involved.

The presence of the fluorescamine derivative of muramate on thin-layer plates, as determined by one-dimensional and two-dimensional chromatography with different solvent systems, indicated that there was indeed muramic acid in the brain, liver, and kidney samples. The fluorescamine derivative of muramic acid was well separated from those of other relevant substances such as NANA hydrolysate, glucosamine, and galactosamine (Table 1). As expected, no fluorophore was formed from NANA without prior hydrolysis. When the entire procedure was followed through TLC without any tissue being used, no muramyl fluorophore could be detected. All experiments and controls were carried out in three separate trials.

We established specificity of the spots of putative muramyl fluorophore by two additional approaches: (i) One approach was the release from the suspected muramyl derivative fluorescent spot of formaldehyde by periodate oxidation. The fluorescence spectrum of the test sample was in accordance with that of standard muramic acid (Fig. 2), i.e., that of formaldehyde (5, 16). (ii) The other approach was the β-elimination of D-lactate from the muramyl fluorescamine derivative and the identification of the product obtained. The experimental result is a spectrum of NADH (Fig. 3), as obtained after dehydrogenation of released D-lactate with D-lactate dehydrogenase, employing authentic muramate.

Several control experiments were performed for each of the two additional procedures described above. In each procedure, a crucial component was omitted to establish the validity of the results obtained with the complete system. These are described in the legends to Fig. 2 and 3.

DISCUSSION

It was intimated above that a specific mode of identifying muramyl peptides in mammalian tissues would have some virtue. Few attempts were made in the past to establish the presence of bacterial cell wall components in "foreign" environments. Thus, establishment of the presence of muramyl peptides in rickettsiae involved color reactions for muramate or treatment of the substance with [^{14}C]acetic anhydride and chromatographic demonstration of the specific acetylated product (1, 21). A microquantitative method (to 10 pg) that employs capillary gas chromatography has recently been described, as applied to *Escherichia coli* (25). It appears to be applicable to animal tissues. Methodologies that have been applied to mammalian tissues employed gas-liquid chromatography–mass spectrometry to detect and determine muramate. In experiments on the cause of polyarthritis (4), 20 mg of bacterial cell wall material was injected into a rat. Approximately 30 to 200 μg (120 to 800 nmol) of muramate was detected per g of fresh spleen after about 1 week. Joint tissue contained 0.2 to 1 μg/g of tissue (fresh weight) under the same conditions. No muramate could be detected in control animals that had not received an injection of bacterial cell wall material. Thus, the existence of stores of muramyl compounds in mammalian tissues, without prior administration of bacterial cell wall material, has not previ-

TABLE 1. R_f values for fluorophors of muramic acid and other samples

Sample	R_f in the following solvent systems[a]:	
	A	B
Muramic acid	59	75
Glucosamine	70	78
Galactosamine	67	77
NANA	0	0
Hydrolysate of NANA	48	69

[a] R_f values are expressed as a percentage of the distance from the origin to the front.

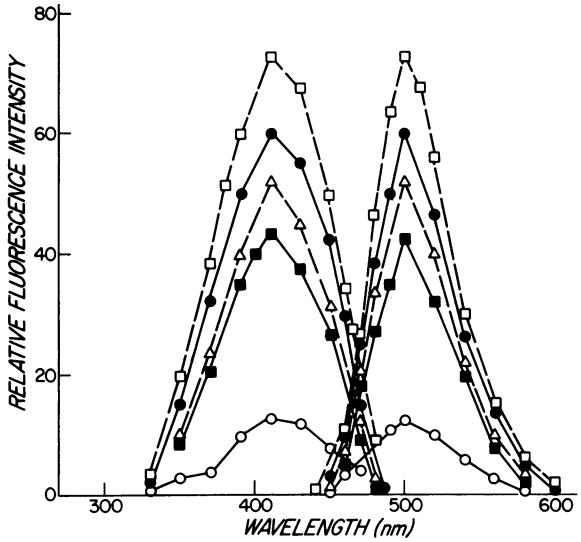

FIG. 2. Fluorescence spectra of formaldehyde derived from muramic acid and the test samples. The excitation pattern is the peak on the left and the emission pattern is the peak on the right; the peaks coincide with those reported for formaldehyde (5, 15). The spectrum of the fluorescamine derivative of authentic muramic acid treated with periodate but without acetyl acetone, or vice versa, showed excitation peaks at 390 nm and emission peaks at 475 nm; both peaks were very sharp. In the absence of muramyl derivative (all other components present), very broad spectra were obtained, and their peaks were <5% the height of standard muramic acid (△). Symbols: △, standard muramic acid (excitation peak, 410 nm; emission peak, 510 nm); □, liver extract; ●, brain extract; ■, kidney extract; ○, silicic acid (plate) blank.

ously been demonstrated, although it is clear that such material can be accumulated (4).

The methodology described here was aimed at exploring the possible presence of muramic acid-containing entities of low molecular weight in animal tissues. In particular, our interest centered on peptidoglycan monomers that, as outlined above, may exert important physiological or pharmacological functions.

Thus, our present system involves the immediate conversion of all muramyl peptides that are extractable into TCA to free muramic acid by hydrolysis. Freed muramate is then converted to a fluorescamine derivative and located by virtue of its movement on TLC plates. To ensure specificity, we were able to apply two chemical methods to the fluorescamine derivative, i.e., the detection of formaldehyde from carbon-6 of the muramate ring and establishment of the release of the D-lactic acid moiety by β-elimination. Of these methods, the latter is more definitive since D-lactate may be expected from plant or bacterial products rather than mammalian sources. In both cases, specific spectra were obtained. Therefore, there seemed little doubt that normal rat tissues do contain muramic acid, presumably as muramyl peptides. We also explored the situation in muscle, and although the data are not as conclusive because of difficulties in obtaining a complete homogenization in TCA, similar results were obtained.

A rough quantitative estimate could be made. It was observed that liver contained the most muramic acid (ca. 100 to 150 pmol/g of tissue [fresh weight]), followed by brain and then kidney. Extract from the tissue of a single rat would suffice for establishment of the presence of muramate, i.e., through the TLC stage. The chemical or enzymatic validation steps require more material (three to four rats). No relevant spot was detectable in the TLC step if tissue was omitted from the process. Thus, muramate was not artifactually acquired.

In the case of liver, the positive finding is entirely predictable because of the large content of Kupffer cells, i.e., macrophages, in the tissue (cf. M. W. Vermeulen and G. R. Gray, Fed. Proc. **42**:1221, 1983). In the case of brain tissue, the situation is of special interest in view of the reported somnogenic or slow-wave sleep-promoting activity of these compounds (10, 11). Factor S, which has been extracted from human urine (10), is a muramyl compound that also contains diaminopimelate. That extracted from brain tissue (9, 19) is probably the same substance, at least as determined by the fact that extracts of both were reported to cross-react with a monoclonal antibody to synthetic muramyl dipeptide attached to a protein (2, 3a).

The reasons for our ability to detect muramic acid in normal rat tissues, in contrast to the report mentioned above (4) that employed gas-liquid chromatography–mass spectrometry, lies in the sensitivities of the methods used. Furthermore, the previous authors dealt only with large molecules; their tissue extracts were subjected to thorough dialysis before analysis (4). On the other hand, as mentioned above, our interest focused on the possible presence of small muramyl peptides, soluble in TCA, which might be involved in such activities as immunoadjuvanticity (13), macrophage stimulation (17, 18), or sleep induction (10). Although our results suggest that various mammalian tissues contain mur-

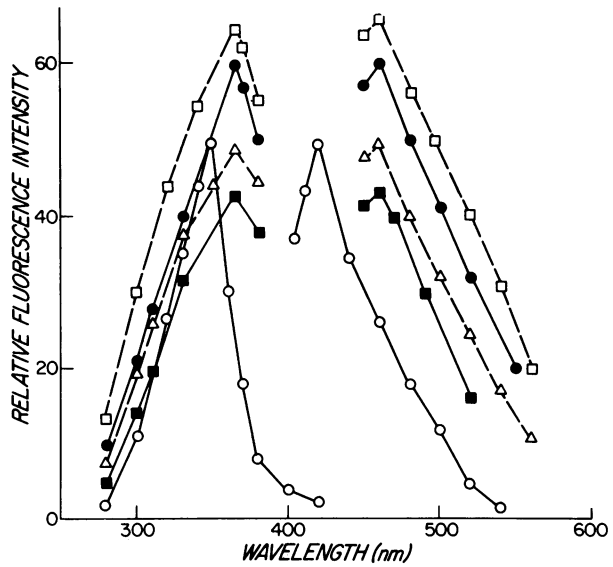

FIG. 3. NADH spectrum after dehydrogenation of products of base treatment of fluorophores with D-lactate dehydrogenase. The excitation pattern is the peak on the left and the emission pattern is the peak on the right; the peaks coincide with those of NADH (15). If the enzyme was omitted (cf. with the standard muramic acid), the excitation peak was at 340 to 350 nm and at the emission pattern peak was at 410 nm. Both spectra were at a level of ca. 5% that of comparable curves for the entire system. If NAD was omitted, the excitation peak was at 335 nm, and the emission peak was at 400 nm. Both curves were sharp, and the relevant peaks were less than 20% of the level of standard muramic acid at those wavelengths. Symbols: △, standard muramic acid (excitation peak, 365 nm; emission peak, 460 nm); □, liver extract; ●, brain extract; ■, kidney extract; ○, blank.

amyl peptides, the modes of accumulation and mobilization and the manner by which the physiological effects mentioned are caused require experimentation.

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