U.S.-JAPAN COOPERATIVE MEDICAL SCIENCE PROGRAM

Program of Leprosy Symposium

INTRODUCTORY REMARKS

The first meeting of the U.S.-Japan Joint Conference was held in October 1965 in Honolulu. Since then the conference has been held in turn once a year in the United States and in Japan. This year the 6th Annual Leprosy Research Conference is being held at the Nippon Toshi Center, Tokyo, 10-12 November 1970.

We are privileged to have 17 participants from the United States and 29 participants from Japan, and in addition 16 members of the Japanese Leprosy Association joined as observers.

There will be 30 papers presented and discussion will follow each. Recently knowledge and technics in the field of immunology have developed remarkably, studies on immunology in leprosy have been carried out more actively by means of these new technics. Consequently, immunology of leprosy became the main subject of this conference. Ten papers concerned with immunology are to be presented. The lively discussions are expected on the behavior of leprous macrophages, cell mediated immunity, humoral and cellular antibodies in leprosy infection and the problem of erythema nodosum leprosum. Other important problems concerning cultivation and identification of M. leprae, experimental transmission of *M. leprae* and chemotherapy of leprosy will be presented and discussed.

As it is considered to be of some reference to the studies on immunology in leprosy, a scientific film "Allergy under Microscope," produced recently, and very highly evaluated among the scientists is to be shown.

Leprosy is truly an international disease called for international efforts, and this fact is recognized in a modest way by these joint leprosy symposia.

We promise that this conference will be even more profitable than those in the past.

Yoshio Yoshie, *Chairman*
Japan Leprosy Panel

Program of the U.S.-Japan Leprosy Panel Conference

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Program of Leprosy Symposium

Nakamura, M. Elongation phenomemon of *Mycobacterium lepraemurium in vitro* and related problems.

The purposes of experiments to be reported here are (1) to study the optimal and reproducible conditions for elongation of *M. lepraemurium* (MLM) *in vitro*, first described by D'Arcy Hart and Valentine in 1963, and (2) to observe the morphologic changes in MLM during long cultivation periods under conditions suitable for elongation.

The methods used and the results obtained are as follows:

The best condition for elongation of MLM *in vitro*. The Hawaiian strain of MLM was used. Different amounts of culture medium (M/15 Sörensen buffer pH 6.0 + additives + 20% bovine serum) were aseptically distributed into the sterile test tubes (10.5 × 1.5 cm.), in order to see if the depth of the medium had any effect on elongation of bacilli. Microscope slides bearing smears were placed in tubes and cultivated for 18 days at 37°C. The results showed that the deeper the medium, the greater was the elongation.

Similar experiments were performed with buffer at pH6, pH7 and pH8 (+ additives and serum) and with Kirchner medium. The results obtained in pH6 medium were also observed in pH7 medium, in which the reaction was physiologic.

Elongation of MLM on the silicon coated slides. The silicon coated slide is suitable for the long-term cultivation of smears of mycobacteria, because the bacilli are not lost from the glass. When MLM were smeared on silicon coated slides and cultivated, the bacilli were observed to elongate, particularly in Kirchner medium. Therefore, it can be said that the silicon slide is suitable for cultivation experiments with MLM.

Transfer cultivation, deep media to shallow media. The idea employed for these experiments was to see whether elongated bacilli divide when transferred from a deep medium to a shallow medium and then to deep medium again. The results obtained were unexpected; multiplication of MLM was observed when MLM was transferred from a deep medium at pH7 to the same medium again. However, these findings are not sufficiently quantitative. Therefore, more quantitative experiments are in progress with an agar block containing MLM.

Oiwa, K. Cultivation of *Mycobacterium lepraemurium* in a metabolically active cell-free medium. Quantitative analysis of the growth of the organism and its reinoculation into mice.

At the meeting of this conference held in 1968, I reported that macroscopic colonies were obtained on a silicone coated glass slide that was incubated at 37°C in a freshly prepared medium containing brain filtrate of young mice, and transferred successively to fresh medium 6 times a week for 32 weeks.

The present report deals with the quantitative analysis of the growth of the bacillus thus obtained and the results of its reinoculation into mice.

Enumeration of the organisms grown on the silcone coated slide was made as follows: 5 µl. of the suspension of Hawaiian strain of murine leprosy bacilli freed of host substance was dropped on a silicone coated slide. Because of the hydrophobic character of the slide, the bacterial suspension does not spread beyond the spot and, after air-drying, the bacteria adhere to the slide to form a disk about 2 mm. in diameter. This kind of slide was used for cultivation in the same way as reported previously.

Every four weeks after inoculation a slide was stained with the Ziehl-Neelsen method. The bacilli were counted in a known area of the disk and the total number of bacilli in the disk was computed.

The generation time of the bacilli was calculated from the number of bacilli in the disk. After repeated experiments it was estimated that the generation time was between 2 and 3 weeks.

The influence of the frequency of transfer to fresh medium was then investigated. When the slide was transferred to fresh medium 3 times a week, the growth of organisms was excellent and comparable to that transferred 6 times a week. When the slide was transferred to fresh medium once a week, no growth was obtained.

The inoculation of the organisms grown *in vitro* into H strain of mice was carried out 4 times. In the first 3 experiments, the inoculum was from a 40 week-old culture, and no lesion was detected even at the site of subcutaneous inoculation. In the fourth experiment, 1.4×10^5 cells per mouse of 20 week-old culture was injected into mice subcutaneously, and small lesions suggesting murine lepromas were recognized 5 months after inoculation.

Matsuo, Y. Attempts to cultivate *Mycobacterium leprae* and *Mycobacterium lepraemurium* in cell culture.

Attempts to cultivate *M. leprae* and *M. lepraemurium* have been carried out in cell-cultures of various sources, including mouse peritoneal macrophages, for several years. Appreciable increases in numbers of bacteria, especially of *M. lepraemurium*, were obtained only in mouse foot pad cell cultures. However, successful multiplication of the organisms in subcultures has not always been observed.

Method. The mouse foot pad cells have been maintained as a monolayer growth in over 60 subcultures so far. The media used were Eagle-HeLa or Eagle MEM containing 2 to 10% calf of fetal calf serum, supplemented with glutamine and penicillin G. Inocula of *M. leprae* and *M. lepraemurium* were suspensions from typical lesions that had been considerably purified by means of trypsin treatment. The bacterial suspension was added to a monolayer culture of the cells and incubated at 30 or 33°C for 72 to 96 hours to allow phagocytosis to occur. At the end of the phagocytosis period, the cells were washed to remove the unphagocytized bacteria. The cells were then suspended in fresh culture medium, transferred to a culture flask and Leighton tubes containing a cover-slip, and incubated at 30 or 33°C. After an appropriate time of incubation, the infected cells in the culture flask were trypsinized, suspended in fresh culture medium twice the volume of the preceeding culture, and transferred to a new culture flask and to Leighton tubes.

Results. (1) *M. lepraemurium.* It seems likely that *M. lepraemurium* often multiplied in the primary cultures. Isolated acid-fast rods and small clusters in different numbers were observed within the cells at the early stage of incubation. Later, intracellular bacteria increased in numbers, elongated in length, became entangled in each other, surrounded the nuclei of the cells and pushed them to one side. Some of the cells were filled with hundreds of bacteria, resembling globi formation. A peculiar pattern of growth, very similar to cord-formation of *M. tuberculosis*, was also observed. Subcultures have been succesful only until the tertiary culture. All attempts to grow the acid-fast bacteria in the cells on artificial culture media have failed. However, subcutaneous inoculations of the materials into mice produced typical lesions like those caused by *M. lepraemurium.*

(2) *M. leprae.* Successful culture of the organism has not yet been obtained. In some instances, bacillary increases, elongation in length. and characteristic arrangements of bacteria, similar to those in cultures of *M. lepraemurium*, were observed. But no evidence of apparent multiplication of bacteria has been obtained so far.

Rightsel, W. A. and Wiygul, W. C. Cultivation studies of *Mycobacterium lepraemurium* in diffusion chambers.

A specialized technic making use of Millipore diffusion chambers was utilized for cultivation studies of *M. lepraemurium.* The cell-impermeable porous chambers were maintained in animals for periods up to 50 days with and without mouse peritoneal macrophages and LM cells. The best yield was a 31-fold increase of bacilli in chambers containing macrophages when maintained in the mouse. However, one of the most promising observations was a one-log increase in the acid-fast bacilli from cell-free chambers maintained in the mouse

for 50 days. There was no doubt that all the chambers maintained in a susceptible host provided greater yields of bacilli than comparable chambers maintained in a nonsusceptible host such as the guinea-pig. In fact, better yields of organisms were obtained when the chambers were maintained on monolayer Petri plate cultures of tissue cells than when held in the guinea-pig. These results indicate that multiplication of *M. lepraemurium* can occur in a cell-free environment within a susceptible host. Also, the studies give evidence that use of the special porous chambers has promising possibility for similar investigations on the cultivation of other fastidious microbes.—[This investigation was supported by the United States-Japan Cooperative Medical Science Program administered by the National Institute of Allergy and Infectious Disease of the National Institutes of Health, Department of Health, Education and Welfare (Grant R22-AI-08051), Bethesda, Maryland 20014]

Ito, T. and Kishi, N. Application of diffusion chamber technic on cultivation of *Mycobacterium lepraemurium.*

The diffusion chamber technic was applied to the cultivation of *M. lepraemurium,* and both *in vivo* and *in vitro* multiplication of *M. lepraemurium* was looked for.

Materials and method. Diffusion chamber: the chamber was prepared by attaching a plexiglass ring (o.d. 14 mm., i.d. 10 mm., depth 2 mm.) and Millipore filters, Type GS (pore size 0.22 $\mu \pm 0.02\ \mu$) with MF cement; the rim of the chamber was carefully covered by MF cement from the outside to prevent outflow of bacilli and invasion of host cells.

Bacillary suspension. The suspension of *M. lepraemurium* was prepared from a mouse subcutaneous leproma caused by the Hawaiian strain.

Host cells. Mouse peritoneal macrophages, mouse kidney cells, LBu cells (thymidinekinase-less mutant of L cells) and guinea-pig peritoneal macrophages were used.

In vitro experiments. A mixture of *M. lepraemurium* or a mixture of bacilli and cells was sealed in the diffusion chambers, and the chambers were inserted in the peritoneal cavity of mice or guinea-pigs.

In vitro experiments. A mixture of *M. lepraemurium* and host cells was sealed in diffusion chambers, and these chambers were placed in rubber stoppered test tubes (i.d. 15 mm., length 8 cm.) and 3 ml. of tissue culture medium was added to each tube. The test tubes were kept at 37°C and the medium was changed twice a week.

Method of observation. The Millipore filter was stained by the Ziehl-hematoxylin-Neelsen method, and the bacilli and host cells were examined microscopically. A stamp smear of the Millipore filter was also examined after Ziehl-Neelsen staining.

Bacillary counting was conducted after the chamber was homogenized with the proper amount of 3% calf serum-PBS until the ring of the chamber was broken into small pieces.

Results.

(1) *In vivo experiments.* (a) No significant multiplication of *M. lepraemurium* was observed in cell-free diffusion chambers inserted in the mouse peritoneal cavity. (b) Logarithmic growth of *M. lepraemurium* was observed when diffusion chambers containing more than 1,000 cells of mouse peritoneal macrophage were inserted in mouse peritoneal cavity. Limited multiplication of mouse peritoneal cells was also observed. (c) Mouse kidney cells multiplied substantially in diffusion chambers inserted in mouse peritoneal cavity, and apparent intracellular multiplication of *M. lepraemurium* was observed in some places of the cell sheet. (d) No significant multiplication of *M. lepraemurium* was observed when diffusion chambers were inserted in the guinea-pig peritoneal cavity.

(2) *In vitro experiments.* (a) Logarithmic growth of *M. lepraemurium* was observed in *in vitro* experiments after a lag phase of about 4 weeks, when the diffusion chambers contained about 10^5 mouse peritoneal macrophages. (b) A medium consisting of 40% horse serum NCTC medium 109 and one consisting of NCTC 109 (50%) horse serum (40%), and a 1:5 dilution of

bovine or chick embryo extract (10%) were effective as maintenance media for mouse peritoneal macrophages.

(3) *Common findings.* (a) Microorganisms that had multiplied either *in vivo* or *in vitro* showed infectivity for mice (b) No multiplication of *M. lepraemurium* in guinea-pig peritoneal macrophages of LBu cells was observed either *in vivo* or *in vitro.*

Ramasoota, T., Dumrongsiri, S., Rassmeeprabha, K., Prasertsaravut, P., Charoenbhakdi, A., Kettanurak, C., Vichien, K., Walter, J., Izumi, S., Okada, S., Uehira, K. and Nishiura, M. Band structures of human leprosy bacilli in Thailand.

The "band structure" of the leprosy bacillus is a peculiar striation which can be seen around the cell bodies. As a result of the previous studies on this structure, it was found that leprosy bacilli have more band structures (Band Index: about 0.8) than other cultivable mycobacteria (Band Index: about 0.2).

As a joint study with the Leprosy Control Division, Ministry of Public Health, Thailand, we studied the band structure of leprosy bacilli obtained from active lepromatous lesions of the patients at the Out-Patient Department of Prapradaeng Leprosarium near Bangkok in three months (from the end of December 1969 to the end of March 1970).

So far, we have collected 36 specimens from 36 active lepromatous lesions. However, only 10 of these specimens proved satisfactory for the electron microscopic analysis of the band structures of leprosy bacilli. The bacilli from each specimen were placed on 5-7 copper grids. and a total of about 60 grids were examined.

After careful clinical study of the patients, we have looked for correlation between the clinical signs of leprosy, especially the speed of the growth of the leproma, and the Band Index of the leprosy bacilli.

The results were as follows: (a) The Band Index differed considerably (from 0.02 to 1.30) in different patients. The value of the Band Index is not constant in each kind of mycobacterium. Between various strains of human leprosy bacillus, variation is as big as that observed between human leprosy bacillus and cultivable mycobacteria. From this finding, it became clear that Band Index cannot be used as a criterion to differentiate between the human leprosy bacillus and other mycobacteria.

(b) Comparative study of the Band Index and clinical symptoms of leprosy, especially the speed of macroscopic growth of lepromas of the patients, seems to tell us that quickly multiplying leprosy bacilli in rapidly growing leproma have a low Band Index; in other words, quickly multiplying leprosy bacilli have fewer band structures than bacilli growing very slowly.

Nishiura, M., Okada, S., Takizawa, H. and Izumi, S. Electron microscope study of metachromatic granules of human leprosy bacilli.

A case of lepromatous leprosy which had never been treated with any antileprosy drugs showed large numbers of leprosy bacilli with distinct metachromatic granules. When the bacilli of this patient were examined by electron microscopy, four different granular structures were found.

(1) Oval electron-dense or electron-transparent large granules (about 300 mμ x 400 mμ). These large granules are found in almost all of the solid bacilli of this patient. They are usually moderately electron-dense, but sometimes electron-transparent. Large granules of this kind are always surrounded with many small foamy electron-dense granules of about 30 mμ in diameter.

(2) Small foamy electron-dense granules (about 30 mμ). This kind of granule is always found around the large oval granules described above. They surround both electron-dense and electron-transparent large oval granules.

(3) Round electron-dense granules (about 90 mμ). This kind of granule is extremely electron-dense. Usually one or two granules of this kind are found in a bacillary cell body. This granule is present almost constantly in any solid bacilli of any patient, and because of this, this structure seems to be a normal organelle of leprosy

bacillus. The function of this granule is not known, but extreme electron-density might suggest the presence of metal compounds in this granule. It is so electron-dense that, even after strong shadow-casting with platinum, it is still visible in the bacillary cell body.

(4) Budding large granules (about 400 mμ). This kind of granule is found outside the bacillary body in a budding arrangement. It seems possible that these budding large granules are the beginning of the branching mode of cell multiplication of leprosy bacilli.

Among these four kinds of granules, only oval large granules (1) and budding large granules 4) are visible with the ordinary light microscope. Small foamy electron-dense granules (2) and round electron-dense granules (3) can not be seen with the light microscope as they are too small. So, the metachromatic granules correspond to these two kinds of visible large granules, i.e., oval large granules (especially oval electron-dense granules) and budding large granules.

As the bacilli with these large granules are usually solid and plump, it is difficult to believe that these bacilli with metachromatic granules are disintegrating, dying leprosy bacilli. Even dividing leprosy bacilli with large granules were found electron-microscopically.

Abe, M. Identification of *Mycobacterium leprae* by fluorescent antibody technics.

Although there have been several reports on the differentiation of mycobacteria by fluorescent antibody technics, none has ever developed to practical use because of wide-spread cross-reactivity of antigens in various mycobacterial strains. The author has demonstrated that the fluorescent antibody prepared from sera of rabbits, immunized with leprosy nodule-extract (NE), stained leprosy bacilli specifically after appropriate absorption procedure, but it did not stain the other mycobacteria at all. Therefore, it seemed possible to identify *M. leprae* with this fluorescent antibody. In order to confirm this fact, the author examined the cross-reactivity of this bacillus with fluorescent antibodies against the other mycobacteria.

The antiserum of rabbit immunized with a crude suspension of leprosy nodules (LS) was used for this experiment, in addition to the anti-NE serum. Antisera against *M. lepraemurium*, BCG, *M. avium*, *M. microti*, *M. fortuitum* and unclassified mycobacteria (p7, p16 and p37) were prepared in rabbits either by intravenous injection of these bacillary suspensions or by multiple injections of the mixture of the suspension and incomplete adjuvant into foot pads and skin. Both methods of immunization were combined in some cases. Purification of IgG globulin fractions from these antisera and labeling with fluorescein isothiocyanate were performed according to the usual methods. The mycobacterial polysaccharides used for the absorption of fluorescent antibodies were extracted from mechanically disintegrated bacillary cells and purified by ammonium sulfate precipitation and starch-block zone--electrophoresis. Smears of *M. leprae* and *M. lepraemurium* were pretreated with carbon tetrachloride and trypsin, while only the former treatment was used in the case of smears of the other mycobacteria.

The results of immunodiffusion tests with anti-NE and anti-LS sera have been reported previously. All of antisera against the mycobacteria other than *M. leprae* gave one or more precipitation lines in agar gel with the corresponding antigen extracted from disintegrated bacilli. These antisera also showed cross reactions with several antigens from the other strains of mycobacteria. Similar results were also observed in the direct staining of bacilli with each of fluorescent antibodies. Therefore, cross reacting antibodies were absorbed by adding polysaccharide fraction prepared from each stain of bacilli. In the case of anti-NE, LS and *M. lepraemurium* fluorescent antibodies, BCG poylsaccharide was used for the absorption. The titers of fluorescent antibodies were scarcely reduced by such absorption procedure. In direct stains of 9 strains of mycobacteria with each of 9 kinds of fluorescent antibodies after absorption, *M. leprae* was well stained with anti-NE and anti-LS but not at all with fluorescent

antibodies against the other mycobacteria. On the contrary, most of the other strains were stained not only with corresponding fluorescent antibody but also with some of the antibodies against the other strains even after absorption of antipolysaccharide antibodies. Such cross reactions did not show any regular relationship with the bacteriologic classification of mycobacteria. The anti-LS fluorescent antibody stained both *M. leprae* and *M. lepraemurium*. This cross reaction would be due to the antibodies which were presumably produced by unknown antigens in crude suspensions of leprosy nodules and not absorbed by the procedure used in this experiment. Except for this cross reaction, anti-LS did not react with 7 strains of cultivable mycobacteria.

These observations seem to reconfirm the previous findings that the common antigen among *M. leprae* and the other mycobacteria was found in the polysaccharide fraction and that the protein antigen in *M. leprae* which reacted with anti-NE and anti-LS was very specific to this bacillus. Accordingly, it may be concluded that identification of *M. leprae* by fluorescent antibody is now made possible.

Drutz, D. J. and Levy, L. The viability of of blood-borne *Mycobacterium leprae*.

Previous studies (Drutz *et al.*, *Clin. Res.* 17 (1969) 365) have demonstrated that lepromatous leprosy is characterized by continuous *M. leprae* bacteremia. The concentration of bacilli in the blood is directly proportionate to the extent of cutaneous bacillary infiltration (skin index) and the solid ratio, and inversely proportional to the duration of therapy. Bacillemia is sparse in patients with erythema nodosum leprosum and absent in tuberculoid leprosy. Crude quantitative studies employing a modified buffy coat smear technic suggested the presence of approximately 10^2 *M. leprae* per ml. of blood in most untreated lepromatous patients. Bacilli were largely intracellular (monocytes and histiocytes; less frequently polymorphonuclear leukocytes) and largely fragmented. Neverthless, the presence of a small proportion of solidly staining bacilli in the blood suggested that a portion of blood-borne *M. leprae* were viable.

Recent work has confirmed the continuous nature of bacteremia in lepromatous leprosy, but more sophisticated enumeration technics suggest that the concentration of bacilli in the blood may be much higher, approaching 10^4 to 10^5 per ml. in one patient with diffuse lepromatosis. Bacilli harvested from the blood of this patient have been demonstrated to proliferate in the mouse foot pad. Results of similar studies in other patients are pending. Nevertheless, it seems likely that at least a portion of leprosy bacilli which enter the circulation are viable, so that those microorganisms which are cleared by reticuloendothelial tissues (liver, spleen, bone marrow) may still be infective. Indeed, a small proportion of leprosy bacilli found in liver biopsy material (Drutz, unpublished observation) of bacteremic lepromatous patients are also solid staining. Although viable bacteria may be delivered to the reticuloendothelial system from the skin, it remains unclear whether *M. leprae* are capable of further proliferation in high-temperature areas such as the liver.

Kusaka, T. Biosynthetic activities of fatty acids in *Mycobacterium lepraemurium*.

It has been known that about the same pattern of fatty acids could be detected in murine leprosy bacilli (MLB) as in many species of cultivable mycobacteria. No investigation, however, concerning the pathway of fatty acid biosynthesis in MLB has been reported. Recently many biochemical publications have revealed that two main biosynthetic pathways of fatty acids can be detected in many organisms; one is a *de novo* synthesis of fatty acids starting from acetyl CoA and malonyl CoA; the other is an elongation of medium or long carbon-chain fatty acids by stepwise condensation with C_2 units (acetyl CoA or malonyl CoA). Applying new micro-technics developed in recent biochemical studies on lipids, we have examined biosynthetic activities of fatty acids in MLB.

Materials and methods. MLB (Hawaiian strain), purified from murine lepromas of mice, were ground cautiously to obtain a

raw whole cellular extract that was used as the enzyme preparation. ^{14}C-labeled short and medium carbon-chain (C_2 - C_{10}) fatty acids were incubated with the enzyme preparation along with the required cofactors; radioactive fatty acids of other organic acids formed after a certain incubation period were detected by chromatographic technics and radiation counts.

Results. It was found that the *de novo* synthetic activity of fatty acids in MLB was almost negligible, whereas the elongation system from decanoic acid appeared to be significantly active; after incubation the radioactivity of 1-^{14}C-decanoic acid was found to be incorporated mainly into normal chain fatty acids with 12, 14, 16, 18 and 20 carbon atoms. These elongations were found to occur by condensation, stepwise, of decanoyl CoA with acetyl CoA. In addition, MLB were found to contain enzymatic activity catalyzing formation of polar organic acids from short carbon-chain fatty acids. Identification of these products is now in progress.

Discussion. Although MLB possess significant ability to form long carbon-chain fatty acids from decanoic acid, they may be unable to synthesize decanoic acid, as shown by their lack of significant activity in *de novo* synthesis of fatty acids. Others have reported that some anaerobic bacteria such as *Selenomonas ruminantium* require for their growth a normal chain fatty acid with 3-10 carbon atoms. It could be reasonably assumed, therefore, that decanoic acid might be considered as one of the nutrients required for the cultivation of MLB *in vitro*.

Speers, W. C., Marley, G. M. and Morrison, N. E. Mycobacterial dihydroflorate reductase. Second sequential blockade site of the *de novo* folate pathway.

It may be argued, *a priori*, that because the multiplication of *Mycobacterium leprae* is inhibited by DDS, sulfadimethoxine, sulformethoxine or sulfamethoxypyridazine, the organism is capable of synthesizing folate coenzymes by the *de novo* pathway. For, if this were not so, *M. leprae* would be capable of utilizing preformed folates present in human or mouse tissues and thus side-track growth inhibition by sulfones or sulfonamides antagonizing the PABA site of the *de novo* folate pathway.

Dihydrofolate reductase represents the second site of the *de novo* folate pathway open to antimetabolite inhibition and is thus of importance in the experimental chemotherapy of leprosy infections. A TPNH-linked dihydrofolate reductase has been found in extracts from a wide range of mycobacteria including *M. abscessus*, *M. borstelense*, and *M. tuberculosis*. The enzyme has been purified from DDS sensitive and resistant mutants of *Mycobacterium sp.* 607. The enzyme is comparable to other bacterial reductases with a molecular weight below 30,000. Substrate specificity studies show that the enzyme is specific for dihydrofolate and does not react with folate or DPNH. Activation does not occur with salts or urea addition.

Active site binding studies with diaminoheterocyclic inhibitors have shown that trimethoprim and pyrimethamine are relatively poor inhibitors of the mycobacterial reductase. This observation thus offers an explanation for the lack of effects of the drugs against *M. leprae* and *M. ulcerans* in the mouse foot pad.—[This investigation was supported in part by the U.S.-Japan Cooperative Medical Science Program administered by the National Institute of Allergy and Infectious Diseases of the National Institutes of Health, Department of Health, Education and Welfare (Grant AI-08416 and Contract NIH-70-2286), Bethesda, Maryland 20014]

Mori, T. and Innami, S. Immune cross-reaction among *Mycobacterium leprae*, BCG and human bone and cartilage.

Sometimes a degradation of finger bones and nose cartilage is seen in lepromatous leprosy patients; in tuberculosis patients vertebral caries are sometimes seen in which the abscess contains no detectable tubercle bacilli. Sometimes a precipitation antibody reacting with the cellular extract of BCG has been identified in the serum of lepromatous leprosy patients, but the detection of this antibody is very difficult in tuberculoid leprosy patients. Since the destruction of finger bone and nose cartilage is seen

only in lepromatous leprosy patients, we speculated that there may be a common antigen among *M. leprae*, BCG and human bone and cartilage. In the present report, a common antigen among *M. leprae*, BCG and human bone has been demonstrated by Ouchterlony's immunodiffusion method and by fluorescent antibody, and inflammation of bone in leprosy and tuberculosis has been discussed as an immuno-cross-reaction.

Methods. ***Sensitization method with M. leprae.*** Since the sensitization of rabbits with *M. leprae* is very difficult, we used a specific method as follows: Freund's incomplete adjuvant, 15 mg. of polysaccharide fraction of *Klebsiella pneumoniae* type 2 and a saline emulsion of 1.1 gm. fresh leprosy leproma were mixed, and 4 ml. injected bilaterally into the gluteus muscles of each of three New Zealand white rabbits. After 40 days a booster injection was given intravenously with 2 ml. of a saline emulsion of 0.5 gm. leprosy leproma. Total blood was collected from carotid one week after booster.

Sensitization method with BCG. A saline emulsion of 45 mg. whole live BCG bacilli cultivated on Sauton medium for 3 weeks, were mixed with Freund's incomplete adjuvant, and 4 ml. injected bilaterally into the gluteus muscles of each of three New Zealand white rabbits. Forty days later booster reinjection was performed intravenously with 1 ml. of a saline emulsion of 1 mg. of whole live BCG bacilli.

Antigen of M. leprae. 3.5 gm. of raw leprosy lepromata were ground with equal weights of quartz sand in a mortar, and made up to 10 ml. with saline; after centrifugation at 10,000 rpm the supernatant was again centrifuged with 40,000 rpm. The supernatant was used as antigen after concentration to one-third the volume.

BCG antigen, M. lepraemurium antigen and bone antigen. These antigens were extracted with water and after centrifugation the supernatants were concentrated with polyethyleneglycol.

Detection method of common antigen. Common antigens were detected by Ouchterlony's agar immune diffusion method.

Preparation method of fluorescent antibody. The gamma-globulin fraction was separated from rabbit antiserum by ammonium sulfate precipitation. One mg. of fluorescein isothiocyanate was added to 100 mg. of gamma-globulin, the free fluorescein isothiocyanate was removed with Sephadex G 25, and over anionic charged fluorescent antibody was removed with DEAE cellulose. Antileprosy fluorescent antibody was treated by absorption with acetone dried human skin powder, and anti-BCG fluorescent antibody was treated by absorption with acetone dried rabbit liver powder.

Results. The water extract of *M. leprae*, BCG, *M. lepraemurium* and human bone made a common precipitation line with antileprosy rabbit serum on the agar plate. The water extract of *M. leprae* and human bone made a common precipitation line with anti-BCG rabbit serum. We could not identify the particular structure of bone which is stained with anti-BCG fluorescent antibody. However, we could detect that cartilage cells and spongy tissue of bone were stained with fluorescent antibody.

Barksdale, W. L. Hansen's bacilli: phenolase, α-N-acetyl galactosaminoyl-galactose, acid-fastness and the pyridine soluble "layer."

Our work has demonstrated that on the basis of the chemical structure of their cell walls, bacteria, isolated in various parts of the world from lepromatous and tuberculoid leprosy, may be divided into two groups: I and II. The mureins of these two groups are dissimilar, Group II organisms may be further subdivided according to the length of the long chain, α-banched, β-hydroxylated fatty acids of their cell envelopes: i.e., the long chain fatty acids in group IIa organisms have formulas close to $C_{32}H_{64}O_3$ whereas those of IIb are closer to $C_{88}H_{176}O_3$. Prabhakaran and associates have shown that bacilli, *M. leprae*, harvested from lepromata and, particularly, from the spleen(s) of cases of lepromatous leprosy, possess a phenolase which is specifically inhibited by diethyldithiocarbamic acid. Such phenolase activity was found by these authors to be absent from true mycobacteria examined by them. While our

group IIa and IIb organisms exhibit no phenolase activity, group organisms from lepromatous and tuberculoid leprosy possess a phenolase of the kind described by Prabhakaran, Kirchheimer and Harris. Group I organisms also possess certain antigens which specifically react with agglutinins, including isoagglutinins, directed against the human mucopolysaccharide end-groups (antigenic determinants), α-N-acetyl galactosaminoyl-galactose and α-D-galactosyl-galactose. Antigens prepared from organisms of groups I and II elicit responses in the skins of some leprosy patients.

The observation of Campo-Aasen and Convit that acid-hematein-fixing material of *M. leprae* could be removed with pyridine, led Clark Fisher to discover that the acid-fastness but not the Gram positivity of most leprosy bacilli could be removed with pyridine whereas the acid-fastness of true mycobacteria could not. This suggests that most leprosy bacilli are not mycobacteria. Slides will be presented which indicate that leprosy bacilli from most lepromatous tissues lack ultrastructures common to mycobacteria. Also electron micrographs will be shown which indicate a correspondence between ultrastructural markers found in leprosy bacilli in tissue and bacilli cultivated from that tissue.

Okada, S. Fine granules in lepra cells of erythema nodosum leprosum.

The electron microscopy of the lesions of erythema nodosum leprosum (ENL) revealed the destruction of some lepra cells including the rupture of intracytoplasmic foamy structure, degeneration of collagen fibers and the phagocytosis of cell debris and leprosy bacilli into the leukocytes that come into the lesion of ENL, as reported by the author.

In addition to these changes, fine, spherical and electron-dense granules of about 90A in diameter could be found in the cytoplasm of lepra cells in the lesion of ENL. The granules were commonly disseminated in the cytoplasm of lepra cells. In some lepra cells, however, many granules were gathered together and formed a lump adjacent to the wall of foamy structure. Otherwise, specific relation of granules with organella of lepra cells could not be observed. The granules could not always be found in each lepra cell of ENL lesion. In the leukocytes which came to the ENL lesion, the fine granules could not be found. The granules were present in all of 6 ENL lesions taken from 6 patients, although the number of granules was small in one case. The granule could not be found in any of 4 lepromata of 4 new lepromatous cases not yet treated. However, in one leproma out of 3 taken from 3 cases under treatment, the granules could be found.

The nature of these granules is still unknown.

Mayama, A. Complement factors in lepromatous patients with erythema nodosum leprosum.

In the previous reports on humoral antibodies in lepromatous leprosy patients with erythema nodosum leprosum (ENL), it was found that the appearance of ENL during the course of antileprosy chemotherapy is caused by self antigen-antibody reactions. In cases of advanced lepromatous leprosy, specially in those of systemic or diffuse forms. the delayed hypersensitivity to the degenerated leprosy bacilli and their phospholipid components (antigens) may cross-react to antigens from human heart, blood vessels, nerve tissues and joints, and may be an important source of intrinsic factors leading to the production of many self-antibodies.

The purpose of this report is to present the biologic characterization of complement factors of serum protein in ENL. Immunofluorescent staining was performed on sections of biopsy specimens from ENL lesions in the skin of 4 lepromatous patients, and in controls from lepromatous skin lesions of 3 patients without ENL. In the indirect incubation method, the antibodies labeled with fluorescein isothiocyanate to the first and third components of complement in the serum were prepared respectively in rabbits. Sera of 10 lepromatous leprosy patients with ENL, 10 others without ENL and of 5 tuberculoid leprosy pa-

tients were examined. Sections were also stained with hematoxylin-eosin and Van Gieson's and Fite-Faraco's methods.

Prominent fluorescence was observed in the cytoplasm of small lymphocytes, plasma cells, histiocytes, and polymorphonuclears at the site of eruptions of ENL only when the sections were treated with the sera of ENL. Granular fluorescent deposits of the third component were much more prominent than those of the first component as demonstrated by fluorescence microscopy in the areas of dermis containing perivascular cell infiltration and/or fibrinoid degeneration with partial necrosis of ENL lesions. Binding of antigen-antibody and complement in ENL could be demonstrated by immunofluorescence. However, no granular fluorescent deposits were found in the ENL sections when the sera of lepromatous leprosy without ENL and of tuberculoid leprosy were used for the sections.

In addition, the complement level of sera in leprosy patients was determined by micro-immunoelectrophoresis and immunodiffusion methods. Sera from 13 cases of lepromatous leprosy taken several days after the clinical manifestation of ENL, 22 lepromatous leprosy patients without ENL and from 3 tuberculoid leprosy patients were used in this study. A significant increase in the third component, and a moderate increase in the fourth component of the complement was seen only in the sera of lepromatous leprosy with ENL. The mean values for third component were 260 mg. per 100 ml. of serum in lepromatous leprosy with ENL, 156 mg. in lepromatous leprosy without ENL and 133 mg. in tuberculoid leprosy. Generally, repeated examination revealed a gradual decline of the increased levels of the third component in the cases of ENL which were at the recovery stage. In lepromatous leprosy with recurrent attacks of ENL, however, there was a marked increase of the third component of the complement before the occurrence of the lesions of ENL in the skin.

Bullock, W. E., Evans, P. D. and Filameno, A. R. Impairment of cell mediated immunity by infection with *Mycobacterium lepraemurium*.

Previously, it has been shown that the isohistogenic Lewis strain of rat can be infected successfully with murine leprosy and that such infection will blockade induction of adjuvant arthritis in this animal (Bullock, W. E. and Evans, P. E., *Fed. Proc.* 28 (1969) 564). To further assess the effect of infection by *M. lepraemurium* on cell mediated immunity, we have measured the ability of leprous rats to reject skin homografts made across a major histocompatibility (H-1) barrier. The median survival time (MST) of skin grafts made from Brown Norway (BN) rats ($Ag\text{-}B^3$) to 14 normal Lewis rats ($Ag\text{-}B^1$) was 8.0 ($\pm$ 1.1) days but in 15 infected animals the MST was prolonged to 10.1 ($\pm$ 5.2) days.

Although the MST of BN to Lewis grafts was significantly prolonged ($p < 0.01$), no graft survived beyond 14 days in leprous animals. In other experiments, the ability of leprous rats to develop delayed hypersensitivity to sheep erythrocytes (SRBCS) was measured after sensitization was attempted with 7.5% SRBCS in incomplete Freunds adjuvant. Plethysmographic measurements of changes in paw volume were performed 24 hours after intradermal challenge into the paw with SRBCS. The mean increase in paw volume after SRBCS challenge in 12 sensitized normal rats was 10.7% and 4.5% in 12 leprous rats ($p < 0.05$). Paw volume increase in normal controls that received only the challenge injection of SRBCS was 3.2%. The production of hemagglutinating antibody in response to SRBC sensitization was normal in infected animals. Finally, we have demonstrated unequivocal reduction in the clinical and histologic severity of experimental allergic encephalomyelitis (EAE) in leprous rats as compared with normal controls. Mild EAE was produced in normal rats by immunization with rat spinal cord in incomplete Freunds adjuvant but EAE was attenuated or absent in infected animals. Immunization with spinal cord in adjuvent containing *B. pertussis* produced hyper-acute EAE in 8 of 9 normal rats; only 4 of 10 infected animals were so affected.

The results indicate that chronic intracellular infection by *M. lepraemurium* may cause moderate suppression of cell mediated immunity as a consequence of the infection itself.—[This investigation was supported by Grant AI-07964 from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20014]

Han, S. H., Weiser, R. S. and Lin, P. O. Behavior of leprous macrophages in the macrophage migration inhibition test.

Using buffy coat cells and macrophage migration-inhibition tests with PPD and leprolin we have previously shown that, whereas the migration of tuberculoid macrophages is markedly inhibited, the migration of lepromatous macrophages is only slightly inhibited. Similar results were obtained in experiments in which the tests were conducted with leprous lymphocytes mixed with normal guinea-pig macrophages. Since then we have tested the capacity of leprous macrophages to respond in migration inhibition tests conducted with mixtures of tuberculin-sensitive lymphocytes, PPD, and leprous macrophages. The results of these tests will be reported and discussed.—[This investigation was supported by the U.S.-Japan Cooperative Medical Science Program, administered by the National Institute of Allergy and Infectious Diseases (Grant AI-08211) of the National Institutes of Health, Department of Health, Education and Welfare, Bethesda, Maryland 20014]

Abe, M., Minagawa, F., Yoshino, Y. and Sasaki, N. Application of immunofluorescence to the studies on humoral and cellular antibodies in leprosy.

In the previous investigations, it was demonstrated by immunodiffusion and immunofluorescence that *Mycobacterium leprae* contained a specific protein antigen that was easily separated from leprosy nodule extract (NE). This antigen induced not only Fernández reaction in leprosy patients but also antibody formation in rabbits. Therefore, one may well question whether or not leprosy patients produce antibody against this protein antigen.

Specific staining of *M. leprae* by anti-NE fluorescent antibody was found to be inhibited by unlabeled antibody, an evidence for the specificity of the immunofluorescence. Such an inhibition tests was applied to detect humoral antibodies in leprosy patients. A smear of a suspension of leprosy bacilli was covered with serum diluted in 1:40 or more, incubated at 37°C for 30 minutes and, after washing, stained with anti-NE fluorescent antibody. No inhibition of immunofluorescence was found with sera from healthy persons, rabbits immunized with BCG or cardiolipin combined with methylated bovine serum albumin, or with sera from 6 cases of tuberculoid leprosy. However, sera from 10 of 12 lepromatous patients with or without ENL showed positive inhibition with serum dilutions of 1:40 or more. Accordingly, it may be considered that patients with lepromatous leprosy produce antiprotein antibody against *M. leprae*, in addition to antipolysaccharide and anticardiolipin antibodies, whereas patients with tuberculoid leprosy produce little or none of these antibodies.

On the other hand, as represented by lepromin reactions, the production of cellular antibody in leprosy seemed to be generally in reverse relationship with humoral antibody formation. This might be based upon the balanced production of two types of antibodies, i.e., predominantly humoral in lepromatous leprosy, and mainly cellular in tuberculoid. However, could the same antigen induce different responses of antibody-forming cells according to the internal environment of the host? In order to clarify these problems, the authors conducted immunohistologic examinations on cutaneous reactions of guinea-pigs sensitized with BCG, using fluorescein-labeled lepromin and fluorescent antibodies against guinea-pigs' lymphocytes, peritoneal macrophages and serum globulins, respectively, and, at the same time, investigated the effect of these antibodies on cutaneous reactions to lepromin. Labeling of lepromin with fluorescein isothiocyanate through cellulose membranes caused no change in the potency of lepromin. Intraperitoneal injec-

tion of antilymphocyte antibody suppressed the cutaneous reactions due to labeled and unlabeled lepromins at both 24 hours and 2 weeks after the injection of antigens, whereas antimacrophage and antiserum globulin antibodies caused no inhibition of the cutaneous reactions. These facts may indicate that lymphoid cells of guinea-pigs sensitized with BCG play a leading part in cutaneous reactions due to lepromin. The results of immunohistologic examination of the skin region will be discussed from the point of view of cellular immunology.

Skinsnes, O. K. Immuno-epidemiologic concepts in leprosy.

The presentation will contrast and compare the historic development of leprosy endemics and epidemics as related to geographic features in several areas of the world, concentrating on the Pacific area. Additional, partialy forgotten, observations on village and home contact spread of leprosy will be presented. From these presentations principles of epidemiology related to the immunopathology of leprosy will be sought and related to problems of leprosy case finding, control and possible eradication.

Fajardo, T. T., Jr. Indeterminate leprosy—a 3-year study. Clinical observations.

Of 54 outpatients diagnosed clinically as indeterminate leprosy on preliminary examination at the Cebu Skin Clinic, Cebu City, Philippines, from 1965 to 1967, 3 (5.6%) were histologically early tuberculoid, and 51 showed indeterminate histopathology. The diagnosis was confirmed by finding acid-fast bacilli in the nerve and/or infiltrate in 42 (82.45%) of the 51 cases.

There was no untreated control group. However, extensive previous experience has shown that an appreciable proportion will fail to continue treatment as outpatients on a voluntary basis, and this shortcoming was made use of in determining the probable effects of varying amounts of DDS therapy.

The present report is a preliminary account of the course of the disease in 27 histologically confirmed indeterminate cases who were observed for at least 3 years, and its relationship to the number and extent of the skin lesions, preliminary lepromin reaction, bacteriologic skin smears and amount of DDS therapy received.

There were 14 males and 13 females; the majority were between the ages of 10 and 25; the duration of skin lesions ranged from a few months to more than 5 years. The lesions, mostly on exposed portion of the extremities, were all macular and hypopigmented; they were generally ill-defined, and all had varying degrees of sensory impairment. In no case was there a history of the lesions having been previously raised above the adjacent skin.

Of the 27 patients, 14 had single lesions of minimal extent, 8 had lesions of moderate extent, and 5 had fairly extensive lesions. In only 1 of the 27 patients were acid-fast bacilli found by the slit skin smears, and then in only one site; however, acid-fast bacilli were noted in all the 27 cases on histopathologic study. Of the 27 patients, 4 adults were initially Mitsuda negative (0 - 2 mm.) (3 converted during the course of observation, 2 were initially doubtful (3 - 4 mm.) both subsequently became positive), and 15 were initially Mitsuda positive (5 mm. & over). In 6 the reactions were not read.

There were 6 transformations noted during the observation period. In 2 patients, their solitary lesions transformed to tuberculoid (reactive) within the first year, after receiving 1.4 gm. and 3.6 gm. of DDS. In 3 patients, tuberculoid transformation was noted in the 1st year (after 12 gm. of DDS), in the 2nd year (after 24.4 gm. of DDS) and in the 3rd year (after 12.0 gm. of DDS), respectively. In one adult patient who was initially Mitsuda negative and who presented a single bacteriologically negative and rather extensive lesion that was clinically and histologically indeterminate, borderline transformation was noted on 2nd year examination (after receiving only 1.4 gm. of DDS), with appearance of new bacteriologically positive widely distributed infiltrated lesions.

In the patients whose lesions became tuberculoid and tuberculoid (reactive), the

lesions subsequently flattened and became inactive or arrested, except in one case who had only recently transformed.

Of 21 patients whose lesions remained clinically indeterminate, the lesions improved moderately in 17 and had become inactive or arrested on last observation (the DDS received so far ranged from 5 gm. to 60 gm. per patient). In 4, the lesions were appraised on last observation as stationary or progressive, after 5.1, 33.8, 41.4 and 46.9 gm. of DDS.

In this preliminary report, the prognostic value of lepromin test was well demonstrated, as well as the expected influence of the extent of initial skin lesions, on the course of the disease. And aside from the probable influence of other factors, clinical improvement was noted with DDS therapy, regardless of the amount received.—[This investigation was supported by the Leonard Wood Memorial and by Grant AI-07266 from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20014]

Abalos, R. Indeterminate leprosy—A three-year study. Histopathologic observations.

Histopathologic studies were made on materials taken from 54 patients who came to the Cebu Skin Clinic, Cebu City, Philippines, for consultation between the years 1965-1967, and whose lesions were diagnosed clinically as indeterminate leprosy. The clinical diagnosis of indeterminate leprosy was confirmed in 42 patients by finding acid-fast bacilli in their tissue section, 9 faied to show acid-fast organisms on serial sections even after thorough and careful search and 3 had tuberculoid leprosy.

The histopathologic changes seen in the 42 cases consisted mainly of round cell infiltration around blood vessels, nerves and dermal appendages; in addition small foci of epithelioid cells were seen in 16 cases. Nerve involvement was perineural, except in 14 cases that also showed endoneural involvement and 2 cases with hyaline change.

Acid-fast bacilli were demonstrated after careful search in nerves in all 42 cases; in 22 cases they were also seen in the infiltrate. In 8 cases, acid-fast bacilli were also noted within arectores pylorum muscles.

Twenty-seven cases were followed histopathologically for a period of 3-5 years. The histologic status of each patient will be discussed and illustrative cases will be presented.—[This investigation was supported by the Leonard Wood Memorial and by Grant AI-07266 from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20014]

Nakamura, K., Hisai, S. and Yogi, Y. Studies on the development of experimental leprosy in mice. The influence of thymectomy, gamma-irradiation, and administration of testosterone.

Multiplication of *Mycobacterium leprae* in the normal mouse foot pad was observed in slow but consistent fashion until the limit of approximately 10^6 bacilli was reached, after which there was no further multiplication. Rees and co-workers have recently reported that the infection of *M. leprae* has produced experimental lepromas (over 10^8 bacilli a footpad) in mice thymectomized and given 900 r of whole body x-irradiation to depress their immunologic capacity, and then some mice were observed microscopic lesions in the footpad. We have observed increased growth of *M. leprae* in the footpads of golden hamsters treated with thymectomy, and thymectomy plus testosterone administration, and also observed a distinctly increased number of bacilli and the development of a nodular swelling characteristic of *M. lepraemurium* in the food pads of mice and hamsters treated with thymectomy, testosterone and chloramphenicol.

The present investigations were carried out to examine the effect of thymectomy, gamma-irradiation (^{60}Co) or administration of chloramphenicol (CM) and testosterone in the thymectomized mouse. Such treatments were employed either alone or in combination.

Thymectomy was performed from 6 to 48 hours after birth (neonatal thymectomy),

or at the age of 4-5 weeks (thymectomy). In addition, some were exposed to gamma-irradiation and transfused with bone marrow. All mice were given commercial food pellets, and in groups 4-7 (described below) CM (100 μ gm./ml.) was added to the drinking water. Testosterone propionate was intramuscularly injected with 0.2 mg./mouse at the 2 week interval after the infection. There were 8 groups of mice, as follows.

1. Neonatal thymectomy.
2. Thymectomy.
3. Thymectomy and testosterone.
4. Thymectomy and CM.
5. Thymectomy, irradiation (a single treatment with 900 r) and CM.
6. Thymectomy, irradiation (twice with 200 r each) and CM.
7. Thymectomy, irradiation (twice with 200 r each), CM and testerone.
8. Controls (foot pad infection only).

In the thymectomized-irradiated mice (group 5), the growth of *M. leprae* during the logarithmic phase of multiplication was not faster than in the controls. However, during the succeeding period, when the growth in the controls had ceased, the growth in the groups 1-7 continued until bacillary counts approached 10^7 - 10^8 approximately 10 months after the infection.

In our study the number of bacilli was smaller than that reported by Rees and co-workers. Possible explanations for the apparent discrepancy include differences in lines of mice or in the administration of chloramphenicol.

However, we have confirmed that thymectomized-irradiated mice show larger numbers of bacilli in the foot pad than untreated mice, and that thymectomy, thymectomy combined with irradiation of low dose x-ray, and administration of testosterone were effective in allowing bacilli to grow at an early stage of the infection.

It may be concluded that our modified methods were superior to the technic of thymectomy plus a whole body x-irradiation. However, further experiments using mice reared in barrier systems must be undertaken to determine whether similar results can be obtained by the method Rees.

Sushida, K. and Hirano, N. Experimental transmission of leprosy bacilli in mice: 1. Inoculation test of human leprosy material into the testis of mice injected with ^{131}I. 2. Clearance test of serum of mice injected with ^{131}I. 3. Study on body temperature of mice injected with ^{131}I.

Experiment 1. Previously, the authors injected human leprosy material into the rabbit testis because it has a lower temperature. This paper deals with the mice injected with sodium iodide (^{131}I, 50 μCi per mouse). One month after the injection of ^{131}I, the thyroid tissue had been disintegrated and there was a fall in body temperature, amounting to 1°C on the average as compared with control mice. The mice were subsequently inoculated with human leprosy bacilli (about 10^5) into the testis.

Results. Eight to nine months after inoculation, acid-fast bacilli in the inoculated site were scattered or gathered extracellularly. About 10-15 months after inoculation, the acid-fast bacilli were found abundantly in the cells, thus forming the globi. These globi were not round like those found in murine leprosy but most of them were spindle shaped. However, the acid-fast bacilli in globi developed into segmented forms 16 months after inoculation. It was considered, therefore, that the functions of the reticuloendothelial system were lowered after ^{131}I injection.

Experiment 2. The effect of ^{131}I on innate immunity of the blood reticuloendothelial system of mice: mice were injected with ^{131}I (50 μCi per mouse), and 3 days, 2 weeks and 5 weeks after injection, the following experiments were carried out. In each experiment 9 mice were used, and 0.1 ml. (10 mg./ml.) of *E. coli* (24 hour culture) was injected intravenously, followed by collections of blood from the heart with syringes containing sodium citrate, 30 and 60 minutes after injection. Of the blood sample obtained from the 3 mice, 0.5 ml. each were pooled. The liver, spleen and testis of each mouse vere emulsified (100 mg./ml.) and the emulsified organs of 3 mice were pooled. Blood and emulsion of organs were diluted with NaCl solution, and the diluted samples were poured into

Petri dishes with melted agar and incubated. The bacterial colonies on the plates were counted. The average number of bacteria in the blood (1 ml.) was compared with that of the control mouse.

Results. The clearance ability of ^{131}I treated mice 3 days after the inoculation was about the same as that of the control mouse; 2 and 5 weeks later a number of bacteria remained in the blood because of diminished clearance. In the testis, the clearance ability was weak, whereas it was unchanged in the liver and spleen.

Experiment 3. The degree of a fall in body temperature and the destruction of thyroid tissue in the mice injected with ^{131}I are not correlated. The authors divided ^{131}I treated mice into two groups. One group contained mice injected with two doses of 100 μCi of ^{131}I, and the second group was injected with 50 μCi three times. In the ^{131}I treated mice, murine leprosy material and human leprosy material were inoculated, and the correlation of the body temperature, thyroid tissue and their lepromas were investigated.

Shepard, C. C. and McRae, D. H. A hereditary characteristic that varies among isolates of *Mycobacterium leprae*.

Isolates of *M. leprae* in mouse foot pads were found to differ in two related properties, the average rate of growth between inoculation and harvest (G) and the number of bacilli in the harvest (H). For "fast" strains, the median values for G were less than 25 days/generation and the median values for H above $10^{6.1}$. For "slow" strains, the median values for G were above 30 and the median values for H were below $10^{5.6}$. Data were available for 59 isolates, and they formed a continuous spectrum between the two extremes. There was no correlation with DDS resistance. The "fastness" characteristic was stable; it did not change on passage in mice, and was in agreement when more than one isolate had been made from the same patient.

No important differences were apparent according to geographic origin of the patient's infection. Histologic studies showed that "fast" strains grew to a higher level before inducing the infiltrate of lymphocytes and macrophages that appears at the end of the logarithmic phase of growth in mouse footpads.

Some of the differences between "fast" and "slow" strains suggest the lepromatous-borderline differences seen in human leprosy. Accordingly, correlation with the type of disease in the patient from whom the strain was isolated is being searched for. However, no correlation has yet been found.

Tsutsumi, S., Sakamoto, Y., Gidoh, S., Nakamura, K., Hisai, S. and Yogi, Y. Screening of various compounds untested to leprosy by mouse foot pad method.

Twenty weeks after the transmission of the SH-O strain ($2.4. \times 10^3$) to both of the hind foot pads of the experimental mice (ddY/F, female), the administration of drugs was started and it was continued for 7 weeks.

The content of drugs in the diet was 0.005%, corresponding approximately to a weekly dose of 60 mg./kg. Numerous compounds were given by injection (synthetic compounds, intramuscularly[1] 60 mg./kg.; vancomycin and novobiocin, intravenously 30 mg./kg.; the other less toxic antibiotics, intramuscularly 30 mg./kg.; the more toxic antibiotics,[2] intramuscularly 6 mg./kg. once a week). The standards were DDS (0.005% in the diet) in the case of synthetic compounds, and rifampicin (30 mg./kg. per week) in the case of antibiotics. The growth of leprosy bacilli in the untreated mice could hardly be detected at 24 weeks, but it was clearly present at 28 weeks. The mice were sacrificed mainly at 28 and 32 weeks.

In the following results "complete" means complete inhibition; +, partially effective; —, ineffective; and "promotive," more growth than in the control. The two readings refer to observations at 28 and 32 weeks, respectively.

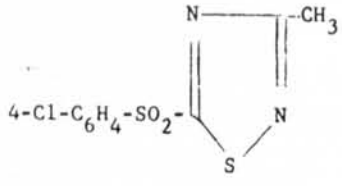
I. Synthetic compounds: DDS (complete, +)

1. Sulfones

4-NH_2-C_6H_4-SO_2-C_6H_4-R-4′

R: COOH (+, +), $NHCH_2COOH$ (+, +), OC_4H_9-n (−, +), $NHCOC_5H_{11}$-n* (complete, complete), $NHCOC_{15}H_{31}$* (complete, +).

DEDDS (complete, +), DDS 2-sulfonamide (−, −),



(complete, -)

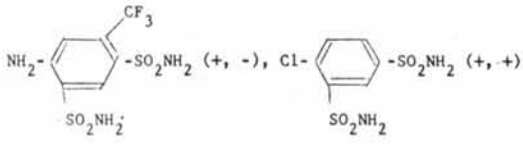
2. Sulfonamides

4-NH_2-C_6H_4-SO_2-R

R: 4-NH_2-C_6H_4-NH- (+, +), 4-n-C_4H_9O-C_6H_4-NH- (−, +), 4-n-C_4H_9O-C_6H_4-$NHCH_2CH_2$- (+, +), 3-CF_3-C_6H_4-NH-[1]

(promotive, promotive), 

-NH- (+, +)[1],

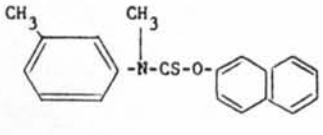


3. Thioureas and rhodanides

4-R-C_6H_4-NHCS-R′

R,R′: NH_2, 4-NH_2-C_6H_4-NH- (complete, −), i-$C_5H_{11}O$, $C_6H_5C\equiv C$- (complete, −); Cl, $C_6H_4C\equiv C$- (+, −)

4-AcNH-R-SCN-1[2]
R: C_6H_4 (+, +), 3-F-C_6H_4 (−, −), 2-F-C_6H_4 (−, promotive), 2-F-C_6H_4* (−, −)



(complete, -)

4. Antituberculosis compounds

INAH (−, −), Pyrazine thioamide (−, −), INAH-$COCH_2NHCSNH_2$[3] (−, −), PAS hydrazide (+, −), α-Phenylmyristic acid* (−, −)

5. Antifungal compounds

Decanoic hydroxamate* (−, −), 3,5-dimethyl-4-nitroso-1-(p-chlorophenyl)-pyrazole* (−, −)

6. Phenazine series[o]

1-CONH-C_6H_4-OC_4H_9-4-n (+, −), 2,3-di-NH_2[4] (complete, −), 1-CH_3O[4] (complete, −), 1,9-di-CH_3O[4] (+, −), 1,6-di-CH_3O[4] (−, −), 1,7-di-CH_3O[4] (+, +), 1,8-di-CH_3O[4] (−, −), 2-CH_3O[4] (+, −), 2-CH_3O, 10-N → O[4] (−, −), 1-Cl[4] (−, −), 1-Cl, 5-N → O[4] (−, −), 1-CH_3O, 8-Cl[4] (complete, promotive), 2-CH_3O, 7-Cl[4] (−, −), 2-CH_3O, 7-Cl, 5-N → O[4] (−, −), 2-CH_3O, 8-Cl, 5-N → O[4] (+, −)

II. Antibiotics:

Rifampicin (almost complete, +)
Esperin** (+, +)
Questiomycin A (−, −)
Mikamycin A (−, −)
Mikamycin A + B** (15:1) (+, +)
Pyridomycin[5] (+, −)
Rufomycin (+, −)
Glumamycin (+, −)
Bottromycin hydrazide[6] (almost complete, −)
o-Carbamyl-d-serine (−, −)
o-Carb.-d-serine + Cycloserine (1:1) (−, −)
Kanendomycin (−, +)
Racemomycine-A purified**[7] (+, +)
Tuberactin (−, +)
Erythromycin (+, −)
Leucomycin (+, −)
Carbomycin (+ almost complete, −)
Amicetin complex (−, −)
Bleomycin** (Promotive, −)
Vancomycin (−, −)
Novobiocin (−, +0)
Coumeromycin (−, −)
Pyrrolnitrin (−, −)
Lincomycin (−, −)
Aureothricin** (−, −)
Tuberin (+, −)
Fusidic acid (−, −)
Usnic acid (+, −)

Generally, sulfones and sulfonamides showed inhibitory effects superior to other series of compounds. Especially, the inhibitory effect of mono-$C_5H_{11}CO$-DDS and DEDDS was found to be more excellent

than that of DDS itself. A new-type sulfonamide, 5-fluoropyridine-2-sulfonamide and, curiously, a diuretic Saltron, showed an inhibitory effect comparable to DDS. In the other series of the synthetic compounds, 4-acetaminophenyl rhodanide and 1,7-dimethoxy phenazine showed an inhibitory effect.

Although the dose of the antibiotics was lower than that of the synthetic compounds, none of the less toxic antibiotics showed an effect comparable to rifampicin. Only partial inhibition could be detected in the case of the basic water-soluble antibiotics. At 28 weeks, Bottromycin hydrazide, Pyridomycin, and macrolides showed an inhibitory effect. However, at 32 weeks their effect could not be detected.

The higher toxic antibiotics such as Esperin, Mikamycin A+B, and Racemomycin-A showed an inhibition comparatively excellent, whereas Bleomycin showed promotive effect.

The numbered compounds were kindly supplied by: (1) Prof. T. Saito of Osaka Pharm. College. (2) Prof. K. Takatori of University. (3) Prof. H. Hisai of Nagoya Municipal University. (4) Prof. I. Yoshioka of Osaka University. (5) Dr. K. Maeda of National Institutes of Health. (6) Prof. S. Nakamura of Hiroshima University. (7) Prof. H. Taniyama of Nagasaki University.

Biggs, J. T., Levy, L., Gordon, G. R., and Peters, J. H. Binding of dapsone (DDS) and monoacetyl dapsone (MADDS) by plasma of animals and man.

Plasma protein binding and plasma acetylation-deacetylation of DDS and its principle human metabolite, MADDS, have been examined in plasma samples from human subjects and several animal species in an attempt to find an animal model which most closely resembles the human. The contribution of protein binding to the variation of the plasma half-lives of DDS and MADDS among species has been assessed. Peters *et al.* (*Fed. Proc.* 29 (1970) 803) have shown that human subjects after ingestion of DDS, establish a MADDS/DDS ratio which is constant for the individual and consistent with rapid or slow acetylation phenotype as determined with isoniazid or sulfamethazine. Maintenance of this ratio as well as the virtual absence of MADDS from human urine could be the function of plasma protein binding.

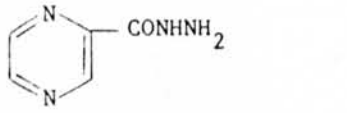
An ultrafiltration technic was used to compare the degree of plasma protein binding of the compounds at a concentration of 2 μ gm./cc. in 1 gm. % protein solution at 25°C. The percentage of DDS and MADDS bound by the various species is shown below:

Species	% DDS Bound	% MADDS Bound
Human	41	97
Dog	31	55
Rat	40	64
Mouse	6	61
Rabbit (slow acetylator)	46	79
Rabbit (rapid acetylator)	46	76

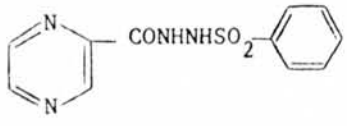
The half-life of DDS is longest in humans, 21 hours., and shortest in rabbits, 1.2 hours, Gordon *et al.* (*Proc. West. Pharmacol. Soc.*, in press). Under the conditions of these experiments, mouse plasma contains a MADDS deacetylase which converts 29 $\pm$ 1% of the MAADS to DDS in 2 hours and 49 $\pm$ 2% to DDS in 4 hours. Deacetylase activity is not exhibited in plasma from humans, dogs, rats, and rabbits under these conditions. These data indicate that species differences in half-life of DDS and MADDS are not solely a function of plasma protein binding, but are dependent on many organ systems in the intact animal. The dog, which has a DDS half-life which most closely resembles the half-life in man, probably is the best model for further study of DDS metabolism. The importance of enterohepatic circulation and renal clearance will be assessed using this model.—[This investigation was supported in part by the U.S.-Japan Cooperative Medical Science Program administered by the National Institute of Allergy and Infectious Diseases of the National Institutes of Health, Department of Health, Education and Welfare (Grants R22 AI-07801 and R22 AI-08214) Bethesda, Maryland 20014]

Satake, Y. The basic studies on chemotherapy of pyrazine derivatives for leprosy. First report.

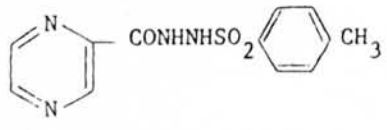
Previously the author synethsized some of pyrazine derivatives, which were designated by the names as follows:



Pycazid



PBS-1



PBS-2

These compounds were investigated with a view to assessing their applications in the treatment of leprosy.

The results of studies on their bactericidal and inhibitory activities against the growth of tubercle bacilli ($H_{37}Rv$) were shown as follows.

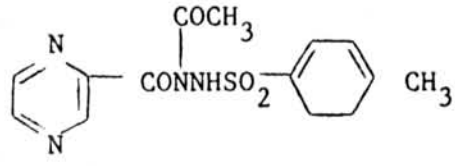
A diluted solution of PBS-1 (10γ) was effective against the growth of $H_{37}Rv$, whereas this dose of Pycazid showed no effect *in vitro*.

PBS-1 and PBS-2 were found to be very weakly toxic as follows.

LD_{50} . . .	Pycazid	150.5 mg./kg.
	PBS-1	1,510.0 mg./kg.
	PBS-2	1,540.0 mg./kg.

The author has accordingly examined pharmacologic functions of these compounds and has obtained the following results:

1. No change of respiratory action and blood pressure even with a large dose.
2. No change of action on the blood vessel of rabbit ear.
3. No action of PBS-1 or PBS-2 on the excised heart of the toad. The heart beat became irregular and stayed in systole with a dose as large as 20 mg./20 ml. of Pycazid.
4. Actions on intestine of rabbit: Pycazid caused spasmogenic activity in a very small dose and decreased the tension of the intestine when used in a large dose.
5. No hemolytic action with any of the three compounds.
6. Identification of the metabolites of PBS-2 could not be accomplished because of the minute quantities present. It is highly probable, however, that the major metabolite has the following chemical structure.



From the results described above, PBS-2 was considered to be a drug for the treatment of leprosy and clinical tests are being carried out.

Tsutsumi, S., Sakamoto, Y. and Gidoh, S. Metabolism of some derivatives of DDS masked by several bioactive radicals.

The understanding of structure-action relationships of sulfone chemotherapy of leprosy remains a difficult problem.

In this study, a number of DDS derivatives have been synthesized of the type R-DDS, R-MADDS, R-DDS-R, R-DDS-G, and R-DDS-S, in which R was diethylaminoacetyl (E) or morpholinoacetyl (M), G the N-glucuronide, and S the N-sulfate. The derivatives are designated DEDDS, DMDDS, etc. The glucuronides and sulfates were hygroscopic, so that they could be identified only chromatographically. ^{35}S-DEDDS and -DMDDS were prepared, and, together with ^{35}S-DADDS and ^{35}S- and ^{3}H-DDS of high specific activity, were used for metabolic studies.

The metabolic disposition of these compounds was studied in mice and rabbits by radio-tracer technics, and their distributions in mice were followed by whole-body autoradiography (SBARG). WBARG showed that ^{3}H- and ^{35}S-DDS were universally distributed—to the tissues, organs and even to brain—5 hours after an intramuscular injection. For the disubstituted compounds, fecal excretion was predominant; there was less distribution into the tissues whereas accumulation in the liver was noted. The decreased radioactivity of mouse sections was greatest for

DMDDS, intermediate for DADDS, and least for DEDDS. In the case of DEDDS concentration of the radioactivity was noted in the region of the eyeballs and in an area mainly corresponding to the submaxillary and sublingual glands for as long as 72 hours after administration.

The plasma levels of DEDDS and of DMDDS in rabbits were markedly higher than those of DADDS. The main metabolic pathway of DEDDS was found to be unmasking of both amino-groups, whereas that of DMDDS was unmasking of only one amino group. Several N-conjugates of DDS could also be detected. An asymetrically distributed DDS derivative (enntoyl MADDS) was found to undergo unmasking of both amino groups by examination of urine after oral administration of the compound to rabbits.

DEDDS was found to inhibit multiplication of *Mycobacterium leprae* in the mouse foot pad. It is not certain, however, whether these long-acting compounds act directly or only after unmasking of the amino groups, especially after oral administration. Further studies of these long-acting DDS derivatives are in progress.

Levy, L., Peters, J. H., Gordon, G. R., Gelber, R., Biggs, J. T., and Tolentino, J. G. Dapsone acetylation polymorphism in man.

The genetic polymorphism for isoniazid (INH) acetylation has been long established by studies of twins, family pedigrees, and population groups. Sulfamethazine (SMZ) is another drug for which an acetylation polymorphism has been established in man. The possibility that dapsone (DDS) might also be polymorphically acetylated in man has been recently investigated in our laboratories.

Employing fluorometric technics for the assay of DDS and monoacetyl dapsone (MADDS), a modified Bratton-Marshall assay for SMZ and acetylsulfamethazine (AcSMZ), and standard fluorometric technics for INH assay, a parallel has been found among the acetylation capacities for INH, SMZ, and DDS in 20 subjects, demonstrating that DDS is acetylated polymorphically. In an extension of this study, 50 Filipino subjects were studied with both SMZ and DDS; a good correlation between acetylation capacities for the two drugs was noted.

Additional studies of DDS and MADDS disposition in small groups of subjects have demonstrated the following: (1) MADDS is readily deacetylated; (2) plasma clearance rates of both DDS and MADDS are the same regardless of the acetylator status of the subject and of which compound is ingested; (3) only a negligible proportion of the dose ($\leq$1%) is excreted in the urine as MAADS and acid-labile MADDS during the first 24 hours after DDS administration; (4) the proportion of the dose excreted as MADDS cannot be used routinely to characterize individual subjects; (5) only the plasma concentration ratio of MADDS/DDS can be used to characterize individuals as "rapid or "slow" acetylators; (6) the characteristic ratio is attained within minutes after ingestion of DDS; (7) this measure of acetylation capacity for DDS has been shown to be a reproducible characteristic of the individual.—[This investigation was supported in part by the U.S.-Japan Cooperative Medical Science Program administered by the National Institute of Allergy and Infectious Diseases of the National Institutes of Health, Department of Health, Education and Welfare (Grants R22 AI-07801 and R22 AI-08214), Bethesda, Maryland 20014]

Peters, J. H., Gordon, G. R., Biggs, J. T. and Levy, L. The disposition of dapsone and monoacetyldapsone in laboratory animals.

We have examined the metabolic disposition of dapsone (DDS) and monoacetyldapsone (MADDS) in mice, rats, rabbits, dogs, and squirrel and rhesus monkeys to assess their potential as models of man for studies of these drugs.

The number of the different species studied, the dose and route of administration of DDS, and the mean half-time of disappearance (hours) of DDS from the plasma were as follows: 25 mice (BALB/c), 1.0 mg./kg., ip, 2.6; 15 rats (Buffalo), 1.0 mg./kg., ip, 6.0; 12 rabbits (6 rapid and 6 slow acetylators; *Proc. Western*

Pharmacol. Soc., in press, 1970), 1.0 mg./kg., iv, 1.2; 11 rabbits (5 rapid and 6 slow acetylators), 100 mg./kg., iv, 1.9; 2 dogs, 1.0 mg./kg., ip, 14.5; 6 squirrel monkeys, 1.0 mg./kg., iv, 5.8; and 6 rhesus monkeys, 1.0 mg./kg., iv, 1.8. Similarly, results obtained after MADDS administration were as follows: 25 mice, 1.2 mg./kg., ip, no MADDS detected; 15 rats, 1.2 mg./kg., ip, 4.3; 14 rabbits (6 rapid and 8 slow acetylators), 1.2 mg./kg., iv, 1.5; 2 dogs, 1.0 mg./kg., ip, 6.8; 6 squirrel monkeys, 1.2 mg./kg., iv, 4.1; and 6 rhesus monkeys, 1.2 mg./kg., iv, 4.2. These half-times of disappearance differ markedly from the values of 20 to 21 hours for both compounds found in rapid and slow acetylator human subjects.

Results of measurements of DDS and MADDS in the plasma of these animals receiving either DDS or MADDS indicated the following: rats, rabbits (depending on phenotype), and squirrel and rhesus monkeys acetylated DDS to MADDS extensively; dogs and mice did not acetylate DDS to MADDS; mice and squirrel monkeys deacetylated MADDS extensively; and rats, rabbits, dogs and rhesus monkeys deacetylated MADDS to DDS only to a limited extent. Man shows different capacities for acetylation of DDS to MADDS depending on phenotype; both phenotypes deacetylate MADDS to DDS extensively.—

[This investigation was supported in part by the U.S.-Japan Medical Science Program administered by the National Institute of Allergy and Infectious Diseases of the National Institutes of Health, Department of Health, Education and Welfare (Grants R22 AI-08214 and R22 AI-07801), Bethesda, Maryland 20014]

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