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BULLETIN OF CHIANG MAI MEDICAL TECHNOLOGY

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NOTES ON MANUSCRIPTS

Review–type articles and case reports are accepted for publication by the Bulletin of Chaing Mai Medical Technology, All manuscripts must be original and should have preferably not been previously submitted to any other publication. Preference is given to material which is of general interest to medical practitioners and research worker in clinical medicine.

Manuscripts must be as concise as possible and should be typed in English with double lind spacing. They should be forwarded to the editor, Bulletin of Chiang Mai Medical Technology, Faculty of Medicine, Chiang Mai University. The title should be limited to a maximum of 10 words and the article broken up with suitable subtitles. Black and White photographs may also be submitted and under special circumstance, colour may be accepted.

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Manuscripts should be arranged in this form :–

An abstract of not more than 100 words containing a brief out–line of the paper must accompany the manuscript.

Introduction.

Material and Methods.

Results of Experiment.

Discussion.

References.

ใบบอกรับเป็นสมาชิก

วารสารเทคนิคการแพทย์ เชียงใหม่

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ถึง บรรณาธิการ วารสารเทคนิคการแพทย์ เชียงใหม่

ข้าพเจ้ายินดีบอกรับเป็นสมาชิก วารสารเทคนิคการแพทย์ เชียงใหม่ ขอได้ส่งวารสารถึงข้าพเจ้า ดังนี้

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BULLETIN OF CHIANG MAI MEDICAL TECHNOLOGY

Vol. 5 No. 3
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Editorial

IMMUNODISC ELECTROPHORESIS AND ITS APPLICATION

By

Sanit Makonkawkeyoon, B.Sc. (Med. Tech.), M.S., Ph.D. *

Before Immunodisc Electrophoresis, a recent developed technic (4), is discussed. It will be worth-while to review shortly about electrophoresis, immunodiffusion, immunoelectrophoresis, and disc electrophoresis to get some ideas about the principles of each technique and how and when these techniques were developed.

1. **Electrophoresis.** Electrophoresis is a method for separating charged molecules in an electrical field. Each charge molecule will move depend upon the total charge of its molecule. If it has positive charge it will move to negative pole (cathode) and if it has negative charge it will move to positive pole (anode).
2. **Immunodiffusion.** Immunodiffusion is a method developed for antigen-antibody reaction in a medium, usually agar. When antigen was introduced into a well in a media (e. g. agar, polyacrylamide gel, starch, etc.) and the antibody in the other well. They will diffuse towards together. At the equivalence zone, a precipitin line will develop.
3. **Immunoelectrophoresis.** Immuncelectrophoresis is a technique developed by Grabar and Williams about twenty years ago. The principle of this technique is the combination of agar-precipitation and electrophoresis in a single experiment.
4. **Dics Electrophoresis.** Dics Electrophoresis was a technique developed by Ornstein and Davis about ten years ago. This technique is widely used to characterized and resolve complex mixtures of proteins such as bacterial toxins, mycotic protein, tissue extracts, purified hormones and enzymes (5). The high resolution of this technique is depend upon the molecular sieving and electrophoretic separation which operate simultaneously (3). Polyacry-

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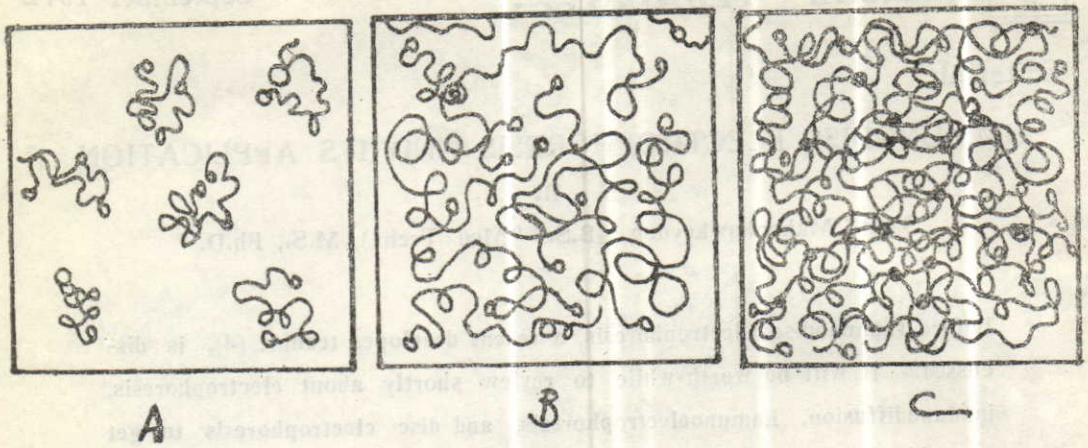


Figure I Schematic representation of the formation of polyacrylamide gel from random gel coils. Transition from a dilute polymer solution (A) through the concentrated solution (B) to the gel (C). -o- cross-linking agent; -o- tie-points.

lamide gel was used in dics electrophoresis because this gels are thermostable, transparent, strong, and relatively inert chemically and can be prepared with a large range of average pore sizes (6).

Polyacrylamide gel is the polymerization and cross-linking product of the monomer acrylamide, CH_2=CH-CO-NH_2, and a cross-linking comonomer, N,N'-methylene-bis-acrylamide (Bis), CH_2=CH-CO-NH-CH_2-NH-CO-CH=CH_2. The three-dimensional network of the gel is formed by cross-linking of polyacrylamide chains growing side-by-side by the mechanism of vinyl polymerization. This leads to the development of numerous, random polymer gel coils (Fig. IA) in which the polyacrylamide chains assume a state of maximum entropy i.e. the most irregular shape. The growing coils move together (Fig. IB) and are cross-linked by main valencies (Fig. IC), where bifunctional compounds, such as N,N'-methylene-bis acrylamide, are built into the polymer chains as cross-linking agents and can react with free functional groups at terminals of other chains. The chemical structure of the gel is shown in Fig. II.

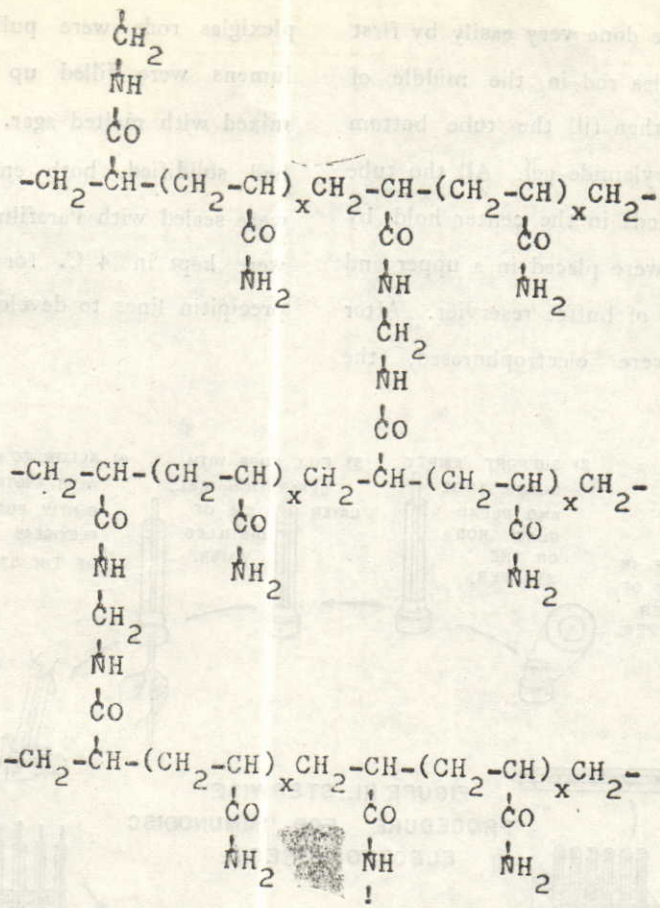
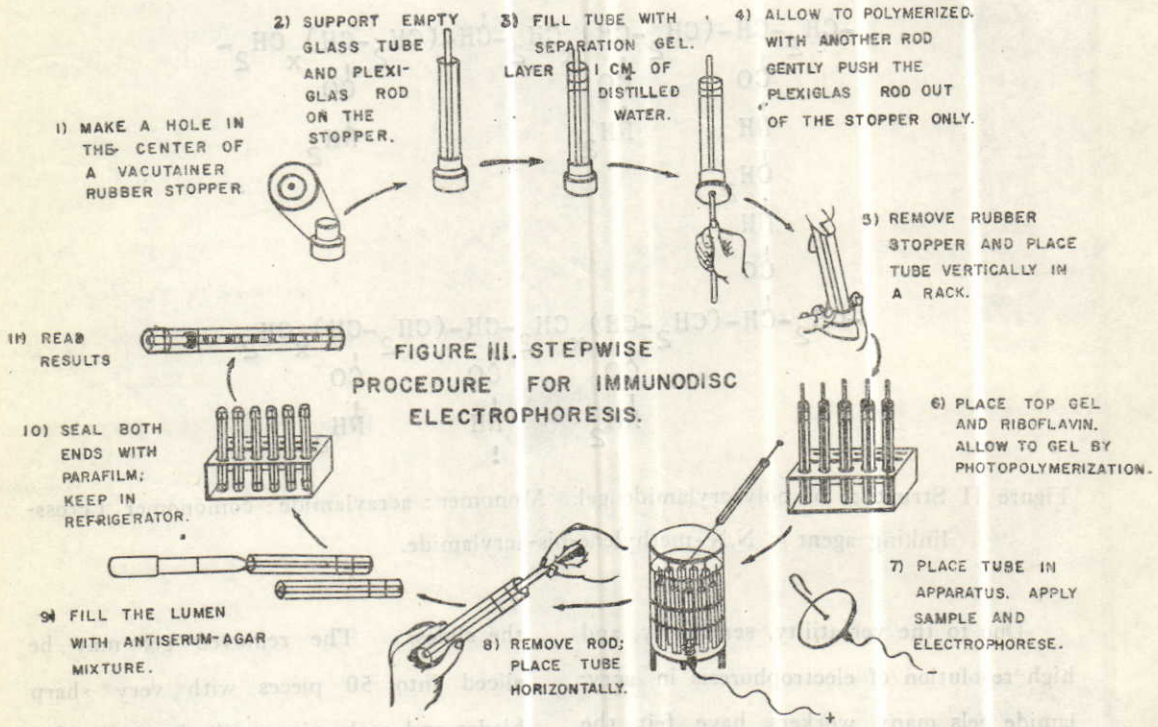


Figure II Structure of polyacrylamide gel. Monomer: acraylamide; comonomer (cross-linking agent): N,N'-methylene-bis-acrylamide.

Due to the versatility, sensitivity, and high resolution of electrophoresis in acrylamide gels many workers have felt the need to extend its use to immunoprecipitation analysis (1,2). The procedures developed so far have required that either the gel be removed from the tube and subsequently embedded in agar to allow the fractions to diffuse and react with the antiserum or the electrophoresis be done in acrylamide gel polymerized directly on the slides. The removed gel may be sliced into 50 pieces with very sharp blades and each piece will be embedded into the agar. Removal of the gel from the tube suffers from the hazard of breakage while the polymerization of the crylamide on the slides requires incubation under carbon dioxide. Therefore, a new technique, called immunodisc electrophoresis, was developed in order to circumvents all such complications. This

technique can be done very easily by first putting a plexiglas rod in the middle of the glass tube, then fill the tube bottom up with polyacrylamide gel. All the tube with plexiglas rods in the center hold by acrylamide gel were placed in a upper and lower chambers of buffer reservior. After the samples were electrophoresed, the plexiglas rods were pulled out and the lumens were filled up with antiserum mixed with melted agar. When the agar had solidified, both ends of the tubes were sealed with Parafilm and the tubes were kept in 4 C. for 5—10 days for precipitin lines to develop (Fig. III).



The immunodisc electrophoresis technique reported here circumvents all such complications as it allows for the precipitin reaction to occur in the electrophoresis tube itself. The technique also has a high resolving power as it yields a large number of precipitin bands than the other techniques (see below). Furthermore since the bands are not in the form of overlaping arcs but appear as discs they are easy to enumerate. These precipitin discs appear in the inner agar column rather than in acrylamide gel. This finding was surprising but it may

be due to the very small pore size of 7.5 per cent polyacrylamide gel which Ornstein (6) reported to be of the order of 50 A.

The use of a hollow cylinder of acrylamide gel as described in this technique makes the disc electrophoresis technique very versatile as it opens a multitude of possibilities for analyzing the samples. For instance, in simple disc electrophoresis the gel can now be stained directly in the tube by filling the lumen with the stain, thus avoiding the need of removing the gel from the tube. The lumen can also be filled with various enzyme substrates contained in agar and the enzymic activities of the isolated fractions can thus be visualized. Presently the technique is based on simple one-way immunodiffusion analogous to Oudin's method. The technique can be easily modified and used for double-diffusion studies if desired. When compare immunodisc electrophoresis, immunoelectrophoresis and disc electrophoresis using the same samples antigen and antiserum. Immunodisc electrophoresis is about 3 times more sensitive than immunoelectrophosis. This method combines the resolving power of disc electrophoresis with the immunological identification of immunoelectrophoresis. It should be very useful to identify antigenic composition of bacteria, fungus, biological materials and especially viruses. When any viral components were isolate, they can be proved to be antigenic of not by this new method. This method should, therefore, find wide application in the antigenic analysis of biologically complex mixtures.

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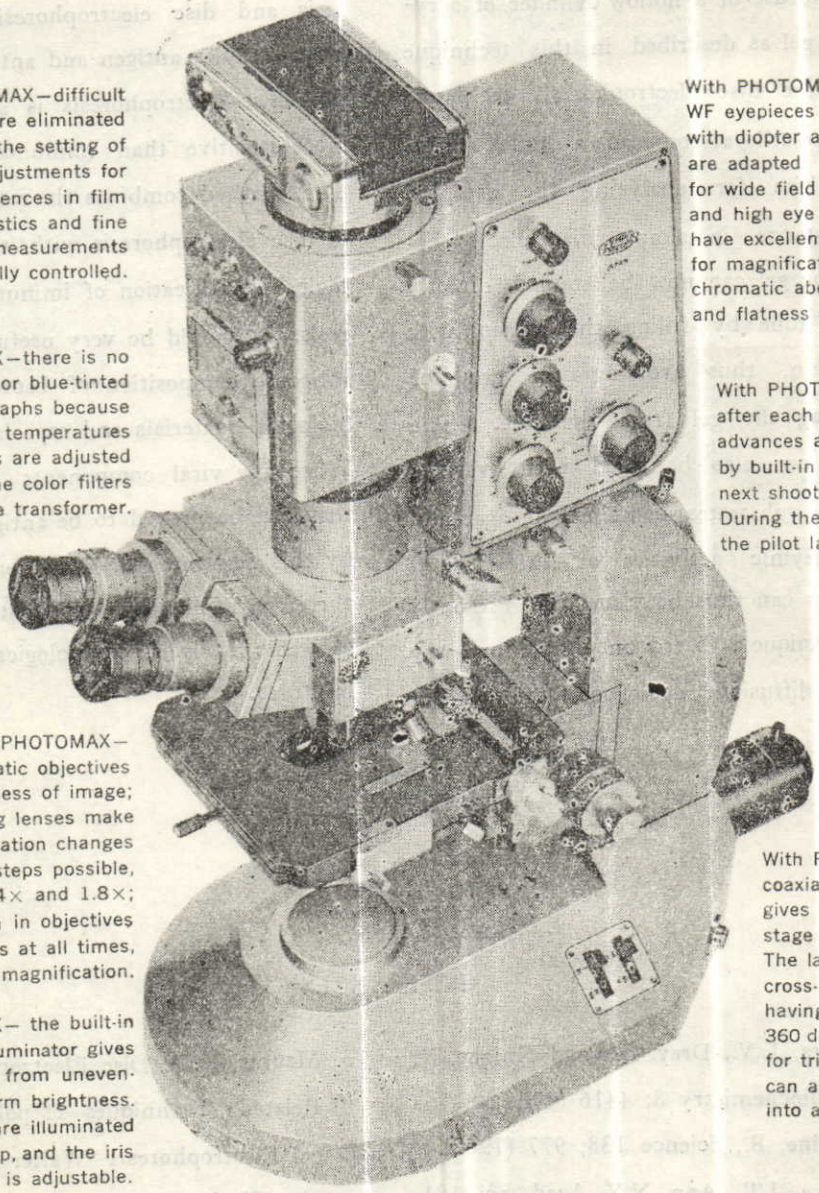

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PHYTOHEMAGGLUTININ-INDUCED BLASTIC TRANSFORMATION AND DNA SYNTHESIS OF LYMPHOCYTE CULTURE

I. LYMPHOBLASTIC TRANSFORMATION

II. TRITIATED THYMIDINE INCORPORATION

By

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When blood leukocytes are incubated in an artificial medium over several days, they exhibit minimal or no growth at all. The granulocytes degenerated within a few days. while monocytes spread out on the surface of the culture vessel, assumming the appearance of cells indistinguishable from tissue macrophages. The majority of lymphocytes remain viable and morphologically unchanged. If the plant lectin, phytohemagglutinin (PHA), is added to the incubating medium, a sequence of dramatic morphorogic changes is initiated. The small lymphocyte undergoes transformation into a larges blast-like cell. (1-12) This morphological changes is accompanied by increased protein, RNA and DNA syntheses, (3, 4, 9, 10, 12) and is followed eventually by mitosis of the tansformed cells.

Lymphocytes can be stimulated in vitro by a wide variety of agents including viruses, bacteria, fungi, protozoa, and pollen as well as purified amino acid polymers and drugs. (13) The agents capable of striggering this chain of events may be catagorized as general stimulants (or nonspecific mitogens) and specific stimulants (specific mitogens). The mitogenic is characterized by its strict dependence on the prior sensitization of the cell doner to immunizing antigens, such as tuberculin, (14, 15) penicillin, (16) streptolysin O (17) tetanus and diphtheria toxoid, purtussis, polio virus and smallpox vaccines, (16, 18, 19) and protein-hapten

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conjugates. (20) These specific mitogens transform only a small portion of lymphocytes, usually 5 to 30 percent. Nonspecific mitogens transform 70 to 80 percent of small lymphocytes (21) isolated from normal individual regardless of prior exposure to each particular mitogen. The plant extracts (PHA, (1-12) pokeweed mitogen (22, 23)), antileukocyte sera, (23) antiallotype sera, (25, 26) and bacterial products (Steptolysin S., (17) staphylococcal exotozin, (26)), are well-known examples of nonspecific mitogens. (See Table I.)

TABLE I: MAJOR CHARACTERISTICS OF DIFFERENT TYPES OF MITOGENS

SPECIFIC MITOGENS	NON-SPECIFIC MITOGENS
1. Transform only 5–30% of small lymphocytes.	1. Transform 70 – 80 % of small lymphocytes.
2. Require prior sensitization of the cell doner to the particular mitogen.	2. Do not require prior exposure to each particular mitogen.
3. Examples: Tuberculin, Penicillin, streptolysin O; Tetanus and diphtheria toxoid; pertussis polio virus and smallpox vaccines.	3. Examples: Plant extracts (PHA, pokeweed mitogen), Antileukocyte sera, Antiallotype aera, Bacterial Products (Streptolysin S, Staphylococcal exotoxin) etc.

Because the magnitude of response to specific mitogen correlates closely with the state of hypersensitivity of the donor, it is believed that the vitro phenomenon may have immunologic significance. (28-30) The significance of the transformation induced with a nonspecific agent such as PHA is unclear. However, PHA-induced blasts bear morphologic features in common with the cells transformed by specific antigens, nd with the large pyroninophilic cells that develop during certain cellular immune reactions in vivo. (31) Thus, response to PHA has been used increasingly as a means of determining changes in proliferative protential and possibly functional state of lymphocyte populations in different physiological conditions such as chronic lyphocytic leukemia, (32, 36) Hodgkin's disease, (37-

39) Sarcoidosis (40-42) ataxia telangiectasia, (43, 44) Tymic aplasia, (45) Sjogren's syndrome, (46) and patients receiving cytotoxic therapy. (47) Circulating lymphocytes from patients described above react poorly to PHA and specific antigens.

Various modifications of the culture technique described by Moorhead et al (48) are widely used to culture of lymphocytes. We are describing a method of short-term in vitro culture of lymphocytes. This procedure involve the separation of lyphocytes from blood, and incubation of the cells in an appropriate artificial medium. Many of the steps are similar to those used in other laboratories. It is the reliable, reproducible and the most simple one to perform in order to study the aberration of cell-mediated immunity mechanism.

IN VITRO CULTURE AND STIMULATION OF LYMPHOCYTE

I. THE MORPHOLOGY STUDY

The degree of lymphocyte stimulation may be assessed either by morphologic changes or biosynthetic activities. The morphologic method entails the enumeration of blasts evolved per 1,000 - 2,000 cells, after a suitable incubation period. Cytologic features are best visualized in smear stained by one of the Romanowsky stains such as Wright's, Giemsa or May-Grunwald, but a nuclear stain, actic orcein (16) and acridine orange (49) have also been successfully employed. Cell growth may be assessed in viable culture by phase-contrast microscopy. (5, 50) Certain steps are essential to ensure viability and successful growth of the cultures: the cells should suffer minimum trauma in their preparation; the incubation milieu should provide adequate nutrients at a physiologic pH; and no potentially cytotoxic materials should be present in the system.

Despite its limitations the morphologic method provide the fast and convenient way of assessing lymphocyte stimulation where precise quantitative is not a necessity.

A—MATERIALS AND EQUIPMENTS.

I: MATERIALS

1. Sterile heparin solution (500 units/ml. in pyrogen-free saline for injection).

2. 5-10 ml. sterile syringe (preferably, disposable plastic syringes).

3. Sterile screw cap culture tube (13 × 35 mm) or sterile disposable plastic tubes 12 × 75 mm. with cap.

4. Sterile capillary pipettes.

5. 1 and 5 ml. sterile disposable plastic graduated pippettes.

6. Culture media: Stock TC-199 Earle's Base or MEM Hank's Base (10 x) Steriled by millipore filtration, kept at 4° C.

7. 6% dextran, sterile, (Abbott Lab.) kept. at 4° C.

8. 0.5 M and 0.25 M. Perchloric acid.

9. Penicillin 10,000 units per ml.

10. Phytohemagglutinin (Bacto - Phytohemagglutinin M. 5028, Difco

11. Stock Hepes buffer (Hydroxyethylpiperazine - N - 2 - Ethanesulfuric acid) pH. 8.1

12. Wright's stain.

13. May - Grunwald stain (Harleco, Phila).

14. Giemsa stain (Figher, Fair Lawn, N. J.

15. 0.85 % Na Cl solution, kept at 4° C.

16. Microscope slides.

17. Cover glasses,

18. Permount (Fighes, Fair Lawn, N.J.)

II. EQUIPMENTS

1. Refrigerated centrifuge.
2. A 37° C.
3. A microscope.

II.I WORKING SOLUTIONS.

1. TC. 199-Hepes solution

Stock Earle's Base (10x)	10 ml.
1. N Sodium Hepes, pH 8.1	4 ml.
Penicillin	10,000 units

Sterile deionized distilled water is added to make a total volume of 100 ml.

2. Culture solution (freshly prepared)

	Solution A	Solution B
Plasma or serum	1.00 ml.	1.00 ml.
TC. 199-Hepes solution	3.75 ml.	4.00 ml.
PHA.	0.25 ml.	-

One ml. aliquots of both solutions are transferred to sterile culture tubes.

II. METHODOLOGY.

1. Five ml. of venous blood is drawn into a plastic syringe containing 0.5 ml. of heparin solution (500 units/ml.). This produces or final concentration of about 45 units heparin per ml. blood.

2. One ml. of 6% dextran is added into the syringe.

3. Incubate the syringe in an upright position at 37° C. (or room temperature) for 45-60 minutes.

4. The supernatant is then collected aseptically into a sterile plastic tube by using the bent needle technique.

5. Centrifuge the supernatant at 1,000 rpm, 4° C. for 10 minutes.

6. Remove the supernatant plasma. Most of the lymphocytes are in the cell button.

7. Resuspend the leukocyte cell button in the TC. 199-Hepes buffer to makes a final concentration of 2×10^6 cell per ml. Do the differential leukocyte count and per cent of viable leukocytes (using 0.2% eosin Y.).

8. Transfer 0.5 ml. of leukocyte suspension into 4 culture tubes containing 1 ml. each of culture solution A (2 tubes) or B (2 tubes). The final cell concentration is 1×10^6 leukocytes /1.5 ml. culture fluid.

9. Incubate these tightly stoppered

culture tubes at 37° C. for 72 hours, undisturbed.

10. After that, the total leukocyte count and differential count is done on each sample using Wright's stain or May-Grunwald - Giemsa stain.

II. BIOSYNTHETIC ACTIVITIES — TECHNIQUE

The incorporation ef ^{14}C or tritiated thymimedine (^{3}H TDR) into a trichloracetic acid precipitate of cells represents the most sensitive indicator of lymphocyte transformation. The parallel study using radioautography has shown that it is the transformed cell which incorporates the thymidine lable. This mothod has the advantage of a low spontaneous incorporation of custimulated small lymphocytes and macrophages do not usually proliferate in short-term cultures. (30) Since it is an ideal technique for detecting those lyphocytes which respond to stimulation by proliferation and following by the proliferation it obviously will not detect lymphocytes which respond to stimulation but do not enter into the S-phase of the cell cycle. Nevertheless, this tachnique has proved to be one of the best way to follow the response of lymphocytes in vitro.

A. MATERIALS AND EQUIPMENTS.

In addition to those required for the morphology study, it requires :

1. Tritiated thymidine (^{3}H - TDR) 10 uc / 20 ug / ml.

2. Hymine hydroxide.

3. Scintillation fluid is prepared as follow :

4.9 gm. of PPO (2, 5-diphenyloxazole)

0.1 gm. of POPOP. (1, 4 - bis (2 - (5-phenyloxazolyl)) - benzene.

120.0 gm. of Naphthalene.

1,000.0 ml. of dioxane.

METHODOLOGY.

In general, the first part of this method is the same as the transformation method described up until step 9. (It is noted that tritiated thymide method may required the leukocyte culture of 48 hours to 72 hours). Then process as follows :

10. After 48 hours of incubation at 37°C., 0.1 ml. of tritiated thymidine solution (10 uc/ml) is added into each culture tubes aseptically

11. Incubate these tubes for an additional 18 hours at 37°C.

12. Do the leukocyte count and differential count, then centrifuge at 800 - 1,000 rpm at 4°C. for 10 minutes. Discard the supernatant fluid in a special container.

13. Wash the cell button twice with 2.0 ml. (or more) of cold saline solution.

14. Add 1.0 ml. of cold 0.5 M. Perchloric acid (PCA) to the cell button, mixed.

15. Incubate the cell suspension at 4°

C., overnight (non-stoppered).

16. Wash the precipitate formed overnight with cold 0.25 M. PCA then allow to dry in air (room temperature) for an additional 1 hour.

17. Dissolve the moist residue in 0.5 ml. Hyamine. Added 0.2 ml. aliquots of this solution (in duplicate) to 14.8 ml. of the PPO and POPOP scintillant.

18. The radioactivity (beta emission) is measured in a Tri-carb Liquid Scientillation Spectrometer (Packed Instrument Co.).

TECHNICAL COMMENTS

1. Although heparin has been reported to inhibit the lymphocyte transformation response, many workers observed no such problem with heparin from Mann Research Lab., N.Y., and Connaught Research Lab., Toronto, especially when used at about 20 units/ml. of blood. It is avisable that heparin used should not contain phenol as a preservative. Heparin preserved with benzyl alcohol is also satisfactory for this purpose. Various doses of heparin have been employed ranging from 20 to 100 units/ml. of blood. The most widely used is 50 units/ml. of blood.

2. A fasting donor is not required, however, grossly lipemic plasma should be avoided. The donor's condition and drug administration must be considered in interpreting the ability and inability of his lymphocytes to proliferate in vitro. These include the upper respiratory tract infection, adrenal cortical steroid or cytotoxic therapy. Oral contraceptive has not been found to interfere with the proliferation of lymphocyte in vitro.

3. The preparation of a "pure" or "relatively pure" lymphocyte suspension generally requires the isolation of the leukocyte from the whole blood, followed by removal of contaminating monocytes and granulocytes. Other workers prefer leukocytes obtained from defibrinated blood. (51) It is either allowed to settle at 37°C. for 45 - 60 minutes or is contrifuged at low speeds (400 - 500 rpm for 10 minutes) to obtain a leukocyte-rich plasma fraction. Because of the low sedimentation rate of the whole blood obtained, dextran, gelatin, fibrinogen or methyl cellulose may then be added before allowed to stand at 37°C. for 30-60 minutes. The addition of such agents will enhance the sedimentation of erythrocytes. The sedimentation rate of heparinized blood at 37°C. is about twice those at 4 C.

4. Purification of the lymphocytes suspension may then be achieved by one of the following techniques :

A. Surface adherence. The leukocyte fraction is incubated at 37°C for 1 to 2 hours on an appropriate surface to which monocytes and polymorphonuclear leukocytes (PMN) attach. The lymphocytes (with contaminating platelets and erythro-

cytes) remain unattached and can be removed virtually free of phagocytes. The materials used for phagocytic attachment are nylon fiber, (52, 53) cotton, (54) (55) glass wool; (23) beads, such as glass (56, 57) and polysterene; (58) or wide-surfaced containers, such as petri dishes. T flaskes, or Blake bottles. (59, 60)

B. Phagocytosis. Carbonyl iron filings added to the leukocyte suspension are ingested only by monocytes and granulocytes. The phagocytic cells then can be removed from the lymphocytes by centrifugation or by introducing a magnet into the cell suspension. (61, 62)

C. **Density gradients.** The leukocytes are sedimented through density gradients made of albumin, (63, 64) dextran, methylcellulose, Ficoll (65) or Isopaque. (66) Phagocytes and red cells, possessing greater specific gravities, sediment to the lower layer, while the less dense lymphocytes remain in the upper portion of the gradient. Some of these methods are time consuming and some albumin preparation may have a stimulating effect on lymphocytes. (63)

5. Centrifugal force in excess of 200× g are not advised. In addition to minimizing possible damage to the cells, gentle centrifugation of the supernatant after red cell sedimentation serves to eliminate most of the platelets. When suspending a centrifuged cell pellet, gentle mixing or trituration with a wide-bore pipette should be employed.

6. It is shown that tissue culture medium 199 supported growth in lymphocyte culture to a much greater extent than MEM. (67, 68) Valentine has found that RPMI 1640, a culture medium developed at the Roswell Park Memorial Institute is excellent for short term lymphocyte culture. (69)

7. In order to obtain a good proliferative response plasma or pooled human sera should be added into the culture medium. Fresh autologous plasma seems to work best (AB plasma, human serum are also adequate). Fetal calf serum is not recommended since it contains a growth-promoting factor that increases background ("spontaneous") transformation. (70) With human lymphocytes, a good response obtained with plasma or serum concentration of between 10% to 20%.

Lymphocyte transformation can be achieved in a serum-free medium, (71) but viability and degree of transformation are greatly reduced. Bergman et al (72) have reported that 6% dextran or 5% glucose can replace serum in the culture medium without diminishing the lymphocyte response to PHA.

8. Penicillin with is included in the culture medium to counteract bacterial

growth, may contribute to the culture response by stimulates the penicillin-sensitized lymphocytes. (16, 70) However, most workers have not experienced this problem.

9. When high concentration of cells are employed cell-to-cell contact will occur earlier than when low concentration are used, It is observed that cell population (or density) must often be at a certain level before cells will grow. In general, concentration of human cells below 0.25×10^6 per ml. should be avoided unless a very small culture vessel is used. With human blood leukocyte cultures $0.25\text{-}0.5\times 10^6$ lymphocytes per ml. will usually give a vigorous response. It is advise that a concentration of 0.5 to 2.5×10^6 lymphocytes per ml. in the original inoculum seems optimum in promoting maximum growth and transformation. (48, 60)

10. The maintenance of a physiologic pH (7.2 to 7.4) is essential for viability and growth of the culture. (73,74) During the final days of culture when considerable cell division occurs, the medium may become quite acid especially when large numbers of cells are employed. It is stressed that the lethal effect of failing to control pH of the medium at the onset would have been likely to effect the lymphocytes before the onset of transformation and later to the transformation cells losses of lymphocytes will occur. (75) The pH of the medium is commonly regulated with a bicarbonate buffer (7.5% solution) and the cell suspensions incubated in tightly stoppered containers (allowing an air phase of at least three times the depth of the fluid) or in lossely covered tubes under an atmosphere of 95% air and 5% CO_2. There is no enhancement of the response whed 7% oxygen is used but the oxygen concentration of greater than 20% (air) will decrease the proliferative response. (76, 77) Many workers find it rather incovenient to use a CO_2-bicarbonate buffer system. A method in which Hepes buffer can be substituted for the two-phase CO_2-bicarbonate system is developed with better results. (78) This single phase liquid buffer used together with medium 199 allowed assay of a mixed lymphocyte culture as early as 2-3 days more stimulation being observed on day 3-4.

11. Optimal growth is also dependent upon the source of lymphocytes and the mitogen used. Lymphocytes from human peripheral blood exhibit good growth and mitosis when stimulated with 0.05 ml. PHA-P per 3×10^6 cells. However, lymphocytes from different tissues vary with respect to the amount of PHA required to induce maximum growth. (79) Poor precision in the past has been attributable to the use of a PHA preparation having a high titer of leukoagglutinin (PHA-P), which

resulted in the formation of large clumps during incubation. The use of purified PHA (such as Burrough-Wellcome "Phytomitogen" MR 68) has greatly increased the precision. (80)

12. One of the initial events is the settling of the suspended cells to the bottom of the culture tube. The formation of this bottom of cells may be of importance for the subsequent immunological reaction. Cell-to cell interaction appears to be a requisite for growth especially during the first 36 hours of culture; (48, 81, 82) hence culture vessels with around or conical bottom with small surface area support better growth than those flat bottom flask or bottle with a large surface area.

13. It is a general practice to eliminate phagocytic cells because of the believe that the presence of the latter may influence the lymphocyte growth kinetic. Conflicting reports have attributed both an enhancing (83) and an inhibitory (84) effect on lymphocyte response to PHA, It is noted that lymphocytes appear to cluster around macrophages in these culture (87) and that if adherent sells are removed from leukocyte suspension the remaining. "Purified" lymphocytes responded poorly to stimulation by antigen and in mixed leukocyte cultures. (86, 88) The transformation response could be restored in the proportion to the number of phagocytes added to these cultures. (85, 86) Although the adherent cells (phagocytes) may be functioning in part as a feeder layer for the lymphocytes, their major function would seem to be in the presentation of antigen to the lymphocytes. However, the role of the macrophage in **PHA-induced** transformation remains largely obscure. "Pure" lymphocyte cultures and unseparated leukocyte cultures achieved a comparable level of transformation in response to PHA, (57, 86) but the possibility can not be ruled out that in "pure" lymphocyte cultures, a fraction of the lymphocytes transform into macrophages, (89. 90) or perhaps a few contaminating monocytes effect the transformation of other cells in the population.

14. Accurate pipetting of the lymphocyte suspension into the triplicate cultures, adequate mixing of the suspension between pipetting are important.

15. The inabillity to count lymphocytes in culture has been due to the aggregation of cells in tight clums by PHA, the accumulation of debris, and the agglutinated red cells (contaminated in the leukocyte suspension) become resistant to the lytic agent in the WBC counting fluid. White cell clumps in PHA-containing culture can be completely dispered by aspirating back and forth 4 to 5 times through a 25 G. disposable needle connected to a 2

ml. syringe, followed immediately by dilution of the aliquot for cell-counting.

Two methods have been described recently for counting aggregated lymphocytes in PHA-containing cultures. (91, 92) Both employ a detergent for disaggregating the leukocyte agglutinates. Stewart and Ingram (91) have found that a proteolytic enzyme, pronase (Calbiochem, L.A., Calif.) is superior to trypsin in digesting nonviable cells and debris and the cytolytic agent, Cetrimide (Cetryltri-methylammonium bromode) in the couting solution (optimum concentration 5 mg/ml.) effectively strips cytoplasm from cells so that accurate counts of nuclei and nuclear volume measurement can be obtained. However, a small number of macrophages often appearing in the cultures may be confused with blast cells. Since degenerating as well as dead cells may be susceptible to the lytic action of pronase, this method raises the added problem of determining what proportion of the cells represented on the smear should be included in the cell count.

Care should be taken to avoid loss of cell clumps above the level of medium.

Accuracy in cell counts may be improved by adding polystyrene particles to the cultures for subsequent identification of the phagocytic macrophages on stained preparations.

16. Other than Wright's stain, 2 other types of staining have been employed. An acid-orcein nuclear stain has proven useful especially in the hands of those accustomed to evaluating nuclear material. Others have experienced some difficulty in confusing macrophage and blast cell nuclei when using this stain and have obtained somewhat higher levels of "blast cells" in unstimulated cultures. Staining with Giemsa as a combination of May Grunwald and Giemsa provides the range of tinctorial properties of cytoplasmic as well as unclear staining which are quite useful in distinguishing cell type.

17. Wilson and Thomson (75) observed that in the absence of PHA, 10% or less of a leukocyte in the culture died in 2 days. Mortality of starting cells is significant as demonstrated by the presence of 150-300 pyknotic small lymphocytes per cu.mm. in 3 day culture. It is advisable to identify these pyknotic cells with non-transformed lymphocytes having died preferentially and progressively, both as a result of alteration in the environment produced by the transformed cells and from toxic action by non mitogenic impurities in the PHA preparation. There ean have been little cytocidal action by the PHA preparation on transformable lymphocytes prior to transformation.

18. With all of these methods one measured only the net result of all substances stimulating the lymphocytes and all

factors inhibiting or limiting their response.

19. The measurement of lymphocyte transformation by tritiated thymidine incorporation is extremely sensitive; however, it is particularly susceptible to errors arising from poor technique. Care with respect to sterility and accuracy must be observed at all stages. Infection in particular can give rise to abnormally high stimulation and therefore any contaminated cultures must be discarded.

REVIEW

Over the past decade considerable interest has been shown in the plant lectin, phytohemagglutinin (PHA), obtained from the red kidney bean - Phaseolus vulgaris. This lectin is a potent erythroagglutinin which is also capable of agglutinating leukocytes and apparently stimulating majority of small lymphocytes (long - live lymphocytes, thymus-dependent) in human peripheral blood culture to transform into large blast-like cells and undergo mitosis. Interest in PHA has been largely due to this latter activity, and numerous reports have documented stimulatory effect in vitro on lymphocyte RNA and DNA synthesis and on morphologic transformation and cell division.

The finding of Tunis (93) of a cytoagglutinin in preparations of PHA which agglutinated all nucleated cells tested indicated that PHA attaches to the surface of a wide variety of cell types. The leukoagglutinating and mitogenic activities of PHA can be separated from its erythroagglutinin by adsorption of the latter onto the surface of erythrocytes, (94-96) and or bentonite. (93) The leukoagglutinin and mitogen have not been separated and appear to reside in the same effective fraction (95, 96) and has in the past led to the conclusion that agglutination is a necessary prerequisite to stimulation. (79)

MECHANISM OF LYMPHOCYTE STIMULATION.

In the resting lymphocyte, RNA and protein syntheses proceed at low levels and DNA replication does not occur. Within minutes of contact, PHA disrupts this quiscent state and in the following few hours the small lymphocyte read justs its metabolism to support the active growth. The mechanism by which nitrogens initiate such profound alteration appears to be mediated in a triggerlike reaction, as washing cells free of the mitogen after only a few minutes exposure does not prevent subsequent transformation. (25, 98, 99) The cell surface has been implicated as the site of the trigger mechanism which becomes activated when PHA interacts when PHA interacts with receptors on the membrane. After a brief exposure to PHA, the electrophoretic mobility of the lymphocytes suggestive of alterations in the surface charge accured. (100) Although the receptors have not been iden-

tified, evidence suggested that they may take the form of gammaglobulin molecules risiding on the membrane. (25) Phospholipids also has been considered as possible surface reactive sites. (101) It has also been noted that lymphiod cells treated with wheat germ lipase as well as trypsin are unable to adsorb antibodies to their surface, thus, the receptor sites binding these cytophitic antibodies may be lipoprotien. (102) Certain sugars on the lymphocyte membrane also may play a role in lymphocyte activation. Fucose and N-acetyl-galactosamine on the membrane play an important role in the "homing" of infused lymphocytes to specific sites in lymphoid tissue. (103) Borberg et al (104) noted that N-acetyl-galactosamine inhibited both the mitogenic activities and the attachment of ^{131}I-PHA to lymphoid cells. This inhibition may due to competition with polysaccharide components at receptor sites on the lymphocyte membrane. An injury-mediated trigger mechanism is also considered as a possible mechanism of action of PHA at the cell membrane.

Enzymes such as trypsin, chymotrypsin and papain promote the transformation of a small percent of lymphocytes, probably by damaging the cell surface. (105) Microwave irradiation, (106) ultrasonic injury (107) and antigen-antibody complex (108) in the presence of complement are capable of stimulating nonsensitized lymphocytes, presumably through injury to the membrane.

Leukoagglutination and increased micropinocytosis that accompany lymphocyte activation is probably not essential to the trigger mechanism. (5) Transformation can be induced by staphylococcal filtrate and antiallotype serum (25) (these agents do not agglutinate lymohocytee while the Vi antigens-coated lymphocytes (thus, are prevented from agglutination) can be activated to from blast cells. (109) Increased micropinocytosis in activated lymphocytes may be secondary only to the membrane changes that have occurred.

Although the cell membrane plays an important role in the activation process, implementation of cell growth most probably proceeds through intra-cellular mechanisms. The controlling site may reside within the nucleus or more likely, the cytoplasm. Hischhorn and Weismann's findings (53, 100) have suggested that the action of PHA may proceed through mobilization of lysomal bound hydrolytic enzymes in the cytoplasm. These enzymes may then proceed into the nucleus for the purpose of stripping DNA of proteinaceous repressor materials that in turn allows for widespread gene activation in terms of DNA-directed RNA synthesis. Drugs such as chloroquin (111,112) and corticosteroids,

(113) which inhibit lymphocyte transformation, may act by stabilizing lysosomal membranes and thus prevent the release of lysosomal hydrolases. (114) Streptolysin S renders lysosomal membranes labile, thereby inducing mitosis in the manner similar to PHA. (17)

Studies utilizing PHA fractions labeled with tritium, (115) 1251. (116), fluorescein isothiocyanate, (117) and mercury show that PHA is localized initially within the cytoplasm. From controlling sites residing in the cytoplasm derepressors may be transmitted to the nucleus.

Direct nuclear activation by PHA remains a distinct possibility. Within minutes after lymphocytes are exposed to PHA, the number of dramatic biochemical events occur in the nucleus. The arginine - rich histones become rapidly acetylated, (119) and the binding of orange to the phosphate groups of DNA is markedly increased. This early nuclear events represent essential steps in gene activation and transcription.

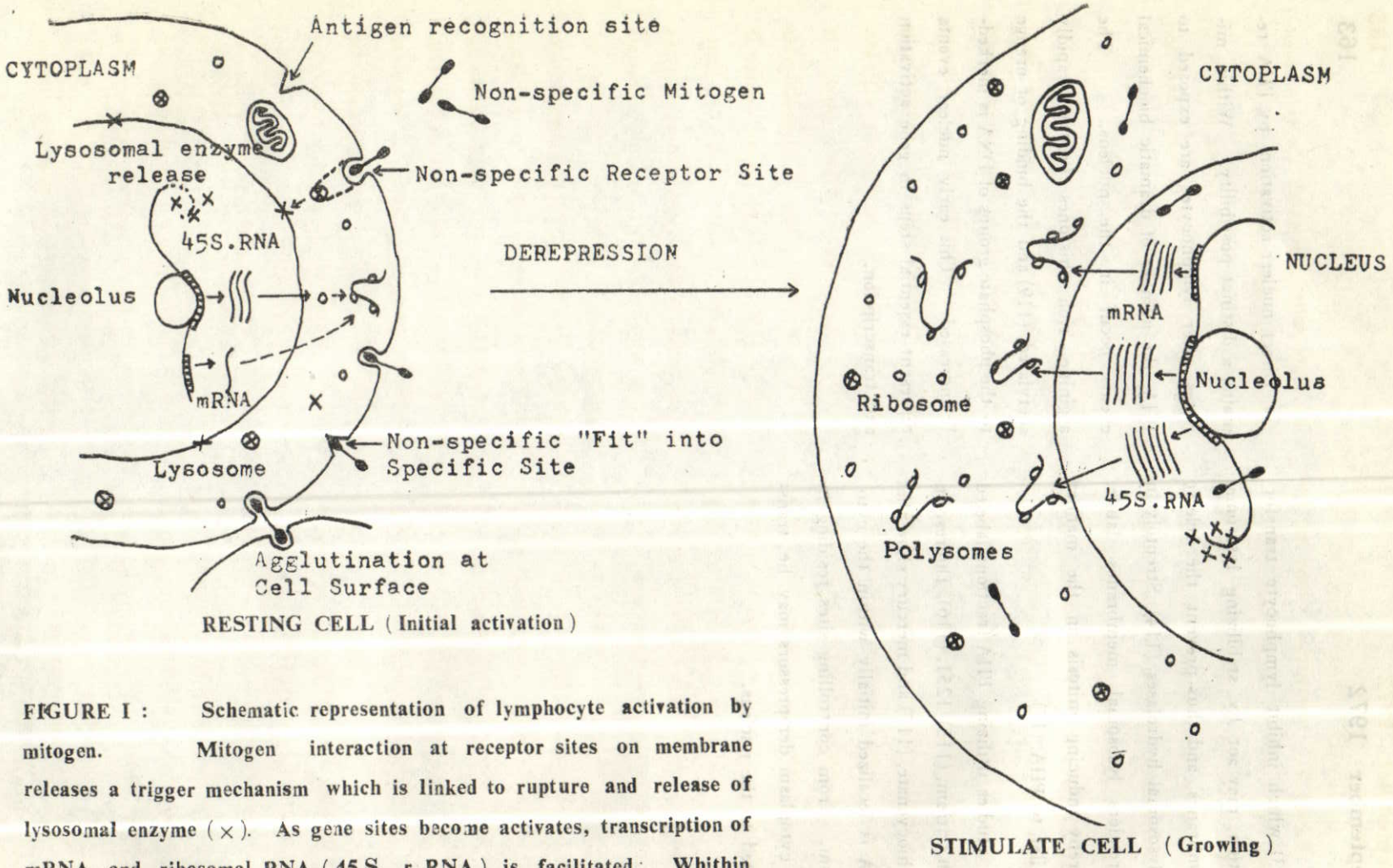


FIGURE I : Schematic representation of lymphocyte activation by mitogen. Mitogen interaction at receptor sites on membrane releases a trigger mechanism which is linked to rupture and release of lysosomal enzyme (×). As gene sites become activates, transcription of mRNA and ribosomal RNA (45 S., r. RNA) is facilitated. Whithin cytophasm ribosomes aggregate along r. RNA strands, now termed polysomes where proteins are synthesized.

Figure I. summarizes some of the views regarding the mechanisms that may operate to control lymphocyte proliferation in vitro. It is not known wheter specific and nonspecific mitogens activate lymphocytes by similar mechanisms. Conceivably, nonspecific agents interact with a wider spectrum of receptor sites, while specific mitogens may engage specific "recognition" sites on the lymphocyte surface. Recent studies indicated that some antigenic determinant sites on the PHA molecule and the lymphocyte surface were shared, as lymphocytes treated with anti-PHA antibodies were unable to respond to PHA (121) There appear to be a growing acceptance that the sequence of events in the normal immune response may commence when antigenic material is phagocytozed by a macrophage. (122) Subsequently, the antigen or antigen fragment is bound nonspecifically to machanism from that of the antigen-induced reaction in which the macrophage plays a crucial role.

METABOLIC CHANGES IN PHA-STIMULATED LYMPHOCYTES

RNA. SYNTHESIS

Acceleration of RNA synthesis has been detected in lymphocyte cultures within 60 minutse after the exposure to PHA. (123) and increased logarithmically for the next 24 hours. It reaches maximal rates between 48 and 72 hours after exposure following which the rate receded toward prestimulation levels by 9 days. (36, 124, 125) As a consequence of PHA stimulation, synthesis of ribosome and non-ribosomal RNA increased.

PROTEIN SYNTHESIS

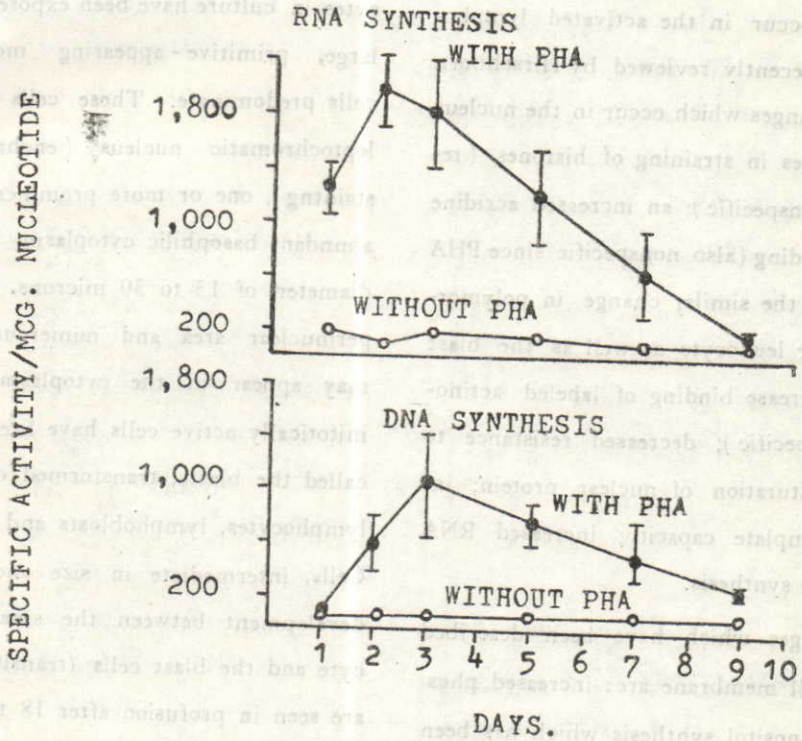
Resting lymphocytes produce small amounts of proteins, commensurate with their quiescent metabolic rate. Following exposure to PHA, protein synthesis is significantly elevated by 3 hours (125) and reaches a maximum at 2 to 3 days. It has been estimated that 10^6 lymphocytes produced about 10 ug of protein (about 1 percent of their weight) within 24 hour. (16) Protein synthesis is essential to lymphocyte transformation, as blast cells fail to develop in the presence of protein inhibitors. (74) Several workers agreed that freshly isolated lymphocytes incorporated labeled amino acids into many classes of protein, haptoglobin, and a variety of substances including all classes of immunoglobulins. (23) PHA-stimulated lymphocytes seem to produce all the varities of proteins synthesized by unstimulated cells, but in greater amounts. Although earlier investigators had identified gamaglobulins as the predominant protein synthesized by PHA-stimulated lymphocytes, most of the recent workers have found that the major protein of these proteins is nonimmunologic. (26, 126, 128, 131, 133) Of the im-

munoglobulins produced, PHA-treated lymphocytes showed either a small increase (131, 132) or no significant increase above the controls. It was postulated that the lymphocytes responding to the nonspecific mitogens must represent a population distinct from that primed to respond to specific antigens. It may be concluded at the present time that PHA (and also the antigen) -stimulated lymphocytes produce vast quantities of protein of which immunoglobulins constitute but a minute fraction. While specific antigens may induce antibody synthesis directly, PHA may enhancing the production of antibodies only in antigen -sensitized lymphocytes in which specific antibody production is already programmed. It is yet to be determined whether PHA does in fact enhance immunoglobulin production in a cell already geared for such synthesis, or whether PHA causes the recruitment of new cells into an immunoglobulin-synthesizing pool.

DNA SYNTHESIS

It appears that only blast cells synthesize DNA and proceed to mitosis. In the circulating blood only a small fraction (less than 0.5 percent) of morphologically small lymphocyte incorporate tritiated thymidine into the DNA. (137, 138) When incubated with PHA, the majority of the cells replicate DNA. Within 18 to 24 hour after exposure to PHA, DNA synthesis remains at a low level, then attain a maximum at 2 to 3 days (Figure 2-3). Almost all of the cells synthesizing DNA have been identified as blast cells on the autoradiograms. Percent transformation and labeling indices show good correlation in these cultures. (139) It is also noted by many workers that even in actively synthesizied cells, the number of cells incorporating thymidine (autoradiograph) is always small than the blast-like cells. This is probably due to the presence of an asynchroncus population of lymphocytes in cultures rather than the possibility of the presence of a factor (s) inhibiting thymidine incorporation. These asynchronous lymphocytes go into s-period (which is rather short period in these cells, approximately 6 to 8 hours) at different times. In addition, it is probably that not all cells that become activated will engage in DNA synthesis and not all blastogenenesis leads to replication. (140) Therefore it depends upon the technique of measuring the thymidine incroporation, the specific activity of tritailed thymidine used, the total amount of thymidine, and the duration of incubation in labeled thymidine, the results may be enormously diferens. (140) The kinetics of RNA syntheses can be investigated by exposing alternate cultures to ^{3}H uridine or ^{3}H-thymidine, respectively. Metabolic response may be

assessed in individual cells by autoradiographic processing of cultures exposed to ^{3}H or ^{14}C-labeled precursors. This technique has the advantage of differentiating cell types that are active in metabolic synthesis and is particularly useful in delineating the pattern in which macromolecular synthesis evolves the cells. (6, 142)



FIGURES 2 AND 3 KINETICS OF NUCLEIC ACID SYNTHESIS BY NORMAL HUMAN LYMPHOCYTES CULTURE IN VITRO.

RELEASE OF LYSOSOMAL ENZYMES. Shortly after stimulation of lymphocytes by mitogens, within 24 hours, there is an increase of lysosomal enzymes. The only enzyme that has been found in supernates following PHA stimulation is acid phophatase. Earlies it was thought that it was released from the cell through toxicity or cell death. It has recently been shown that, following phagocytoses, and concomittant with it, there is release of lysosomal enzymes into the supernate. This is not accompanied by release of other cytoplasmic enzymes, therefore, appears to be relatively specific. Shortly after stimulation of lymphocytes (within 18 minutes) there is an increase of endocytosis, and it was speculated that, similar

to the phagocytosis, there is a release of lysosomal enzymes. (143)

OTHER METABOLIC CHANGES.

These early changes which are observed to occur in the activated lymphocytes are recently reviewed by Hirschhorn (143). Changes which occur in the nucleus are: changes in straining of histones (relatively nonspecific); an increased acridine orange binding (also nonspecific since PHA will cause the similar change in polymorphonuclear leukocyte as well as the blast cells); increase binding of labeled actinomycin (specific); decreased resistance to heat denaturation of nuclear protein; increase template capacity; increased RNA and DNA synthesis.

Changes which have been described in the cell membrane are: increased phosphatidyl inositol synthesis which has been detect as early as 15 minutes after exposure to PHA; increased in phosphatidyl choline and adenyl cyclase activities with an increased in absolute level of cyclic AMC.

MORPHOLOGIC CHANGES ACCOMPANYING LYMPHOCYTE STIMULATION.

Two or three days after the lymphocytes in culture have been exposed to PHA, large, primitive-appearing mononuclear cells predominate. These cells possess a leptochromatic nucleus (enchromatically staintng), one or more prominent nucleoli, abundant basophilic cytoplasm and a cell diameter of 15 to 30 microns. A clear perinuclear area and numerous vacuoles may appear in the cytoplasm. These mitotically active cells have been variously called the blasts, transformed or activated lymphocytes, lymphoblosts and PHA cells. Cells, intermediate in size and cytologic development between the small lymphocyte and the blast cells (transitional cells) are seen in profusion after 18 to 30 hours of culture with PHA, but may be found thoughout the culture period.

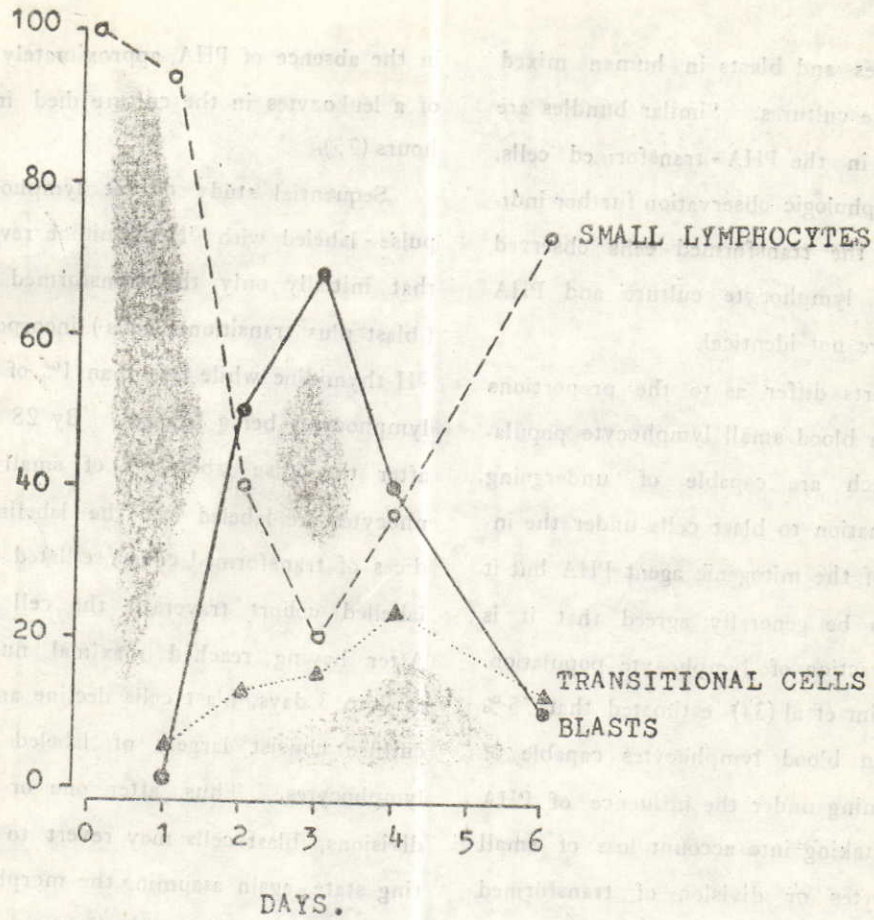


FIGURE 4: PERCENTAGE OF DIFFERENT TYPES OF LYMPHOCYTES IN CULTURE DURING STIMULATION WITH PHA.

The blast cells that develop in PHA-treated cultures exhibit an enormous increase in ribosomes, an hypertrophied Golgi apparatus, and a scant amount of rough-surfaced endoplasmic reticulum. The transformed cells are quite active in micropinocytosis, which accounts for numerous vesicles within the cytoplasm (5). Mitochondria are increased in numbers and many electron-densed acid phosphatase bodies are located in the proximity of the Golgi apparatus. It was observed earlier that both transformed lymphocytes from tuberculin sensitization (144) and the blasts seen in the mixed leukocyte culture (145) exhibit the fine structures that are indistinguishable from those observed in the PHA-transformed cells. Parker et al (146) has recently found that bundles of cytoplasmic fibrils are observed in activated

lymphocytes and blasts in human mixed lymphocyte cultures. Similar bundles are not seen in the PHA-transformed cells. This morphologic observation further indicate that the transformed cells observed in mixed lymphocyte culture and PHA culture are not identical.

Reports differ as to the proportions of human blood small lymphocyte population which are capable of undergoing transformation to blast cells under the influence of the mitogenic agent PHA but it seems to be generally agreed that it is only a fraction of lymphocyte population. Oppenheim et al (34) estimated that 75% of human blood lymphocytes capable of transforming under the influence of PHA without taking into account loss of small lymphocytes or division of transformed cells. However, the lower average value of 60% (45-73%) is observed when loss and gain are also considered. (75) In fact, in the absence of PHA, approximately 10% of a leukocytes in the culture died in 48 hours (75).

Sequential study of rat lymphocytes pulse-labeled with ^{3}H-thymidine revealed that initially only the transformed cells (blast plus transitional cells) incorporated ^{3}H-thymidine while less than 1% of small lymphocytes being labeled. By 28 hours after the pulse label 26% of small lymphocytes are labeled but the labeling indices of transformed cells oscillated as the labelled cohort traversed the cell cycle. After having reached maximal numbers at 2 to 3 days, blast cells decline and the culture consist largely of labeled small lymphocytes. Thus, after one or more divisions, blast cells may revert to a resting state, again assuming the morphologic features of small lymphocytes. These quiescent cells can be restimulated to yield a second spurt of blast development. (148)

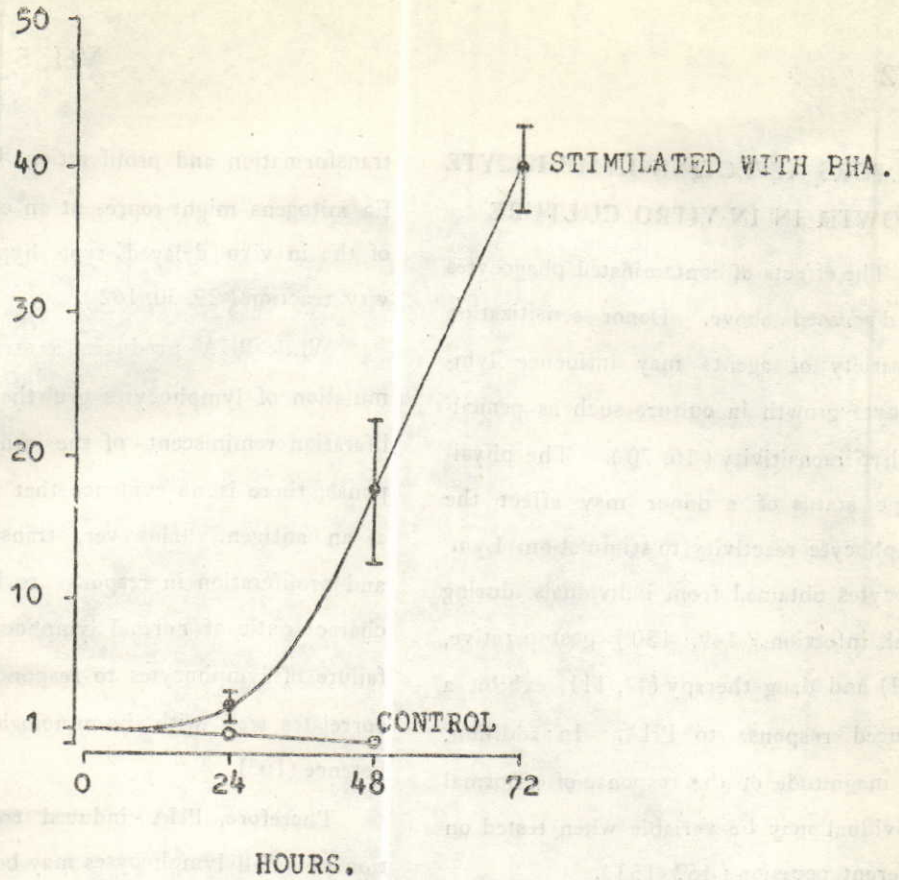


FIGURE 5 : LYMPHOCYTE UPTAKE OF TRITIATED THYMIDINE.

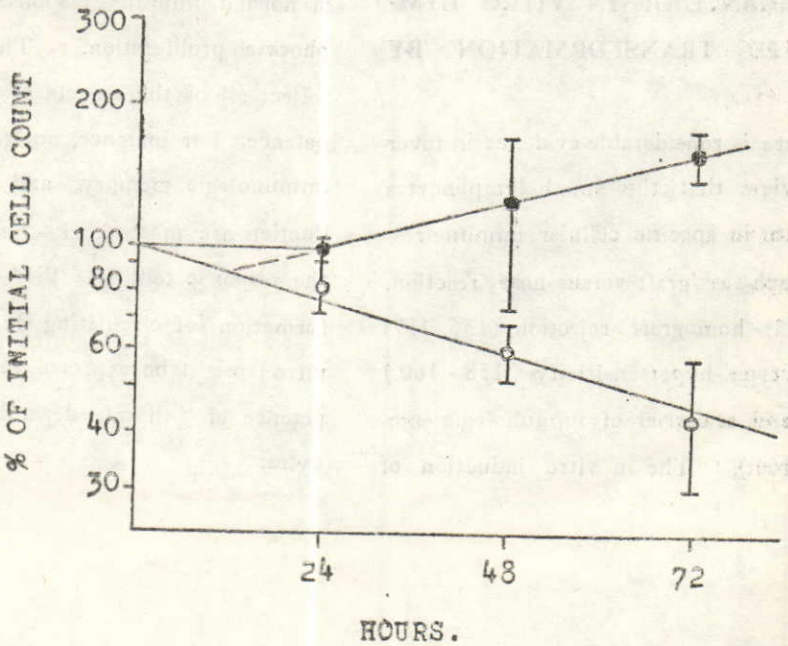


FIGURE 6 : EFFECT OF PHA ON LYMPHOCYTE PROLIFERATION.

FACTORS AFFECTING LYMPHOCYTE GROWTH IN IN-VITRO CULTURE

The effects of contaminated phagocytes are discussed above. Donor sensitization to variety of agents may influence lymphocyte growth in culture such as penicillin hypersensitivity (16, 70). The physiologic status of a donor may affect the lymphocyte reactivity to stimulation. Lymphocytes obtained from individuals during viral infection, (149, 150) postoperative, (151) and drug therapy (47, 111) exhibit a reduced response to PHA. In addition, the magnitude of the response of a normal individual may be variable when tested on different occasion (152. 153).

SIGNIFICANCE OF IN VITRO LYMPHOCYTE TRANSFORMATION BY PHA

There is considerable evidence in favor of the view that the small lymphocytes participate in specific cellular immune response such as graft-versus-host reaction, (154, 155) homograft rejection, (156, 157) delayed type hypersensitivity, (158 - 160) and acts as a carrier of immunologic memory (161). The in vitro induction of transformation and proliferation by specific mitogens might represent an expression of the in vivo delayed type hypersensitivity reaction (29, 30, 162).

While PHA produces a striking stimulation of lymphocytes growth and proliferation reminiscent of the immune response, there is no evidence that PHA acts as an antigen. However, transformation and proliferation in response to PHA is a characteristic of normal lymphocytes, and failure of lymphocytes to respond to PHA correlates well with immunologic incompetence (163).

Therefore, PHA - induced transformation of small lymphocytes may be regarded as an in vitro model of at least one phase of normal immune response-namely lymphocyte proliferation. This would not reflect all of the aspects of immune competence. For instance, antigen recognition, immunologic memory, and antibody production are most likely not reflected in the response to PHA. PHA-induced transformation of circulating lymphocytes (in vitro) might be expected to test the competence of " thymus-dependent " lymphocytes.

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A SURVEY OF THE HELMINTH PARASITES OF FRESH-WATER FISH IN CHIANG MAI PROVINCE

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ABSTRACT

Between November 1971 and February 1972, 12 species of helmimth parasites were recovered from 95 fishes, representing 15 fresh-water species from different areas in Chiang Mai Province: The following helminths are described : 3 species of trematode; **Oreintocreadium** sp, **Acanthostomatid** sp., metacercaria of **Opisthorchis** sp, and one unkonwn metacercaria from **Anabas testudineous.** Four species of nematod, including **Procamalanus** sp, **Camallanus anabantis, Cucullanus** sp. and **Gnathostoma spinigerum** larva. Three species of cestode, **Bothriocepphalus** sp., **Caryophyllaeides** sp. and the sparganum larvae of **Spirometra.** Two species of Acanthocephala; **Pallisentis gaboes**, and **Neoechinorhynchus** sp. Public health implications and effect on commercial fisheries are discussed.

INTRODUCTION

In the northern region of Thailand, edible treshwater fish are an important source of animal protein, and the fisheries industry, provides both work and cash income for thousands, of famllies varous species of fish are raised commercailly, or caught in the rivers, canals and ponds and sold alive in local markets.

Certain northern specially dishes include raw or partially cooked or fermented fish. The methods of preparation are such that several helmith parasites are regularly transmited to man in such dishes as Koi-pla, pla-ra, pla-som, and pla-lab (6). The infective stages of **Opisthorchis viverrini Gnathostoma spinigerum** and **Spirometra mansoni** have all be reported from edible fish (1, 6, 8, 9, 10). A recent survey of 2,000 school children in Chiang Mai city reported 12.6 % positive for **Opisthochis** eggs in the stool.

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Besides the public health aspects of helminth parasites of fishes, endemic disease within the fish population is also a potential treat to the fishing industry itself. Other than the rather informal work of Pearse, 1933 (4), no systematic survey of the helminth fauna of fish in Thailand has been cried out. At the suggestion of the Department of Parasitology, the present survey hopes to contribute more knowledge to this important subject.

Materials and Methods:

During the period of survey 95 fish belong to 8 families, 10 genera and 15 species (TABLE I) were collected at random from stalls in local market and from the Department of Fisheries Station at San Sai northeast of Chiang Mai. Most of the fish from the markets were caught in the Mae Ping or Mae Chem rivers south of Chiang Mai city in Chom Tong district. Most specimens were examined immediately after death, being kept alive in the laboratory in tanks until studied. The external surface gills and mouth were carefully examined for ectoparasites and all organs and tissues were dissected and pressed between glass plates. Attention was given to recording accurate counts of helminth populations found. All data was recorded on accession number cards in the Department of Parasitology, Faculty of Medicine, Chiang Mai University.

Standard methods of fixation, preservation and staining were used: Nematodes were fixed in acetic acid, washed in 70% alcohol with 5% glycerine and evaporated into pure glycerine for mounting and examination. Trematodes were washed first in saline and placed in tap water in the refrigerator to expell the eggs and relax the specimens for fixation; cestodes and acanthocephala were left in the refrigerator until scolex or probosis were everted; both were fixed in hot A. F. A., stained in carmine, dehydrated through alcohols and mounted in permount. Specimens not immediatly examined were stored in labled glass vials in 70% alcohol with 5% glycerine.

RESULTS AND DISCUSSION

Helminth parasites belonging to 12 genera were recovered during the survey (Table 2), one unidentified species of metacercaria was also found. Three helminth found are of know public health importance, i. e. the metacercaria of **Opisthorchis viverini** the infective larva of **Gnathostoma** and the sparganum larva of **Spirometra** sp. All of the other helminths encountered may be presumed to be of some importance in the commercial fisheries industry, although no obvious pathology was noted in the host examined. Measurements are in microns, unless othewise noted.

TREMATODA

Oreintocreadium sp.

Figure 1

Definition : Allocreadiidae Stossich, 1903; Oreintocreadiinae Yamaguti, 1958; **Oreitocreadium** Tubangui, 1931.

Description : (based on 13 fixed and stained specimens) Boby small, elongate, 990 to 1452 (1119) long; spinulate. Oral sucker 110 to 154 (132) in dimeter, simple; prepharynx subterminal; pharynx comparatively large 66 to 88 (74.3) wide. Esophagus short 110 to 154 (127) long; intestinal ceca terminal at posterior extremity. Testes 66 to 110 (93.3) in diameter, in posterior half of body, median, tandem or slightly diagonal, entire. Ovary 60 to 80 (75), median, between acetabulum and anterior tests. External seminal vesicle present. Cirrus pouch clavate, 110 to 198 (176) long, not extending beyond acetabulum posteriorly. Uterus extending as far as posterior extremity, eggs small 18 to 22 by 15 to 18; Vitellaria numerous distributed in lateral fields of hindbody from the ovary anteriorly, to the posterior extremity

Host. Clarius batrachus.

C. macrocephalus.

Anabas testudineus

Habitat. intestine

Discussion : The genotype, **O. batrachoides** Tubangui, 1931, from Luzon and four other species from India have been reported from the same host as in the paesent studies, **C. batrachus.** Two additional species are reported **Channa (Ophicephalus) punctatus,** both from India. Khalil (1961) reported and additional species from **C. lazera** in the Sudan. Cupta (1951) has reviewed the genus and devised a key to the Indian species, but until such time as his paper or type specimens, are available, species diagnosis is not possible. The finding of **Oreintocreadium** in **C. macrocephalus** constitute a new host record, and Thailand, a new range for this parasite.

Acanthostomatid sp.

Figure 2

Description : (2 poorly preserved specimens, fixed and stained). Body elongate 690 to 850 heavily spined. Oral sucker funnel shaped, 66 to 88 wide by 44 to 55 deep, with circumoral crown of spines. Pharynx directly behind oral sucker; esophagus long, bifurcating just anterior to acetabulum. Acetabulum 55 to 66. Testes diagonal, posterior to ovary. Uterus extending posterior to testes; eggs small, 18 to 22.

Host. Clarius batrachus

Habitat. Interestine.

Discussion : The condition of the specimens precludes accurate generic definition and it is described here as an aid

to future workers. Although resembling echinostome flukes, with the characteristic pharynx and spined oral sucker, the fact that few of the former have been reported from fishes, lead the author to tentatively assign the specimen to the family. Acanthostomatidae, Poche, 1926.

Metacercaria of Opisthorchis viverrini

Description : (10 fixed and stained specimens) Body oval shaped, length 330 to 360 (348), width 240 to 260 (248); oral sucker 55 to 66 (62), excretory vessel present, primordia of sex organs present.

Host. Puntius gonionotus

Habitat. Flesh of fins, muscle and beneath scales.

Discussion : Specimens were recovered from the flesh of dorsal, pectoral and tailfins and from beneath body scales. Several hundred metacercaria were usually found. **Opisthorchis** infection in human has long been known in northern Thailand (8) and its distribution in local edible fishes has been recently studied (9).

Unknown metacercaria

Figure 3

Description : Body oval shaped, length 242, width 220; oral sucker 55, ventral sucker 66. Esophagus 88 long from oral sucker to intestinal bifurcation.

Host. Anabas testudineous

Habitat. in the flesh of body.

Discussion : 3 specimens were recovered in the flesh of **Anabas testudineous**, only one specimen is complete.

CESTODA

A total of 62 specimens were obtained from the intestine and body cavity of **C. batrachus** and **C. macrocephalus.** The most abundant was sparganum larva, occuring in 41 % of fish examined. (TABLE 4)

Bothriocephalus sp.

Figure 4

Description : Body small, total length 12 mm, 560 wide at midbody. Scolex elongate 110 long by 55 wide, club-shaped with apical disk, indented on each surfical edge, without hooks. Bothria, shallow, longitudinally elongate. Neck lacking. Strobila with distinct segmentation. Proglottides craspedote, with more or less distinct median furrow and distinct marginal glove.

Host. Clarius batrachus.

Habitat. Intestine.

Discussion : Only one specimens was recovered, immature, without sex organs. Specific indentification was therefore not possible. **Bothriocephalus sp.** are reported from a wide variety of fresh - water fish and marine fish of different families (13).

Caryophyllaeides sp.

Figure 5

Definition : Caryophyllidea Olssen,

1893; Caryophyllaeidae Leuckart, 1878; Caryophyllaeinae Nybelin, 1922.

Description: Body elongate 1.5 to 3.8 (2.6) mm. in total length, width at mid-body, 264 to 374 (300); scolex undifferentiated, Testes numerous, medullary, anterior to ovary and uterus, median field. Cirrus pouch oval, large; cirrus opening in preovarian region. Uterus not extending anterior to cirrus pouch. Ovary "A" shaped.

Host. Clarius macrocephalus,

C. batrochus

Habitat. Intestine

Discussion: Three specimens of this parasite were recovered, one was broken during recovery. This species has been reported from Europe, Russia, Finland, but not in Asia, therefore the occurence of **Caryophyllaeides** in **C. macrocephalus** is a new host record and new range for this parasite in Asia.

Sparganum larvae of **Spirometra** sp.

Description: Body small, total length 3 to 8 (5.2) mm, 350 to 500 (435) wide. Other features unknown.

Host. Clarius batrachus

C. macrocephalus.

Channa striatus

Habitat. Intestine

Discussion: Spargana were found in large numbers in 41% of fishes examined, particularly in **Channa stritaus** and both **Clarius** species (Table 4). When fed to cat, the spargana developed into typical adults of the genus **Spirometra.**

NEMATODA

Camallanus anabantis, Pearse, 1933

Figure 6

Definition: Camallanidae Railliet et Henry, 1915.

Description: Mouth slit-like, buccal capsule consisting of two lateral chitinous valves with longitudinal lip-like thickenings internally, teeth absent. From the point of junction of the valves, dorsally and ventrally, a trident-shaped chitinous process is directed backwards. Esophagus consisting of a short anterior muscular portion and a long posterior glandular portion enlarged posteriorly.

Male: (one adult specimen)

Body lenth 1.94 mm., maximum width 88 at midbody. Esophagus 264 long, maximum width 55 at base; slightly swollen at posterior end. Nesve ring 44 from anterior extremity. Tail length 132 from anus coiled ventrally. Small caudal alae present, about 7 pairs of costiform preanal papilae, 2 pairs of small adanal and a number of postanal papillae, spicules simple, unequal 55 to 67 long.

Female: (4 specimens)

Body length 2.2 to 5.65 (3.43) mm., maximum width 88 to 110 (99) at mid-body. Esophagus 220 to 308 (258) long

and 44 to 66 (53) wide at base. Nerve ring 44 to 55 to 55 (49.5) from anterier end. vulva about midle of body, 1.00 to 2.54 (1.44) mm. long from posterior end; uteri opposed, posterior ovary lacking, viviparous.

Host. Anabas testidineus

Habitat. Stomach.

Discussion : This species has been previously described by Pearse, from the same host, as well as **Clarius batrachus** and **Channa (Ophicephalus) punctatus**, neither of which was found infected in this survey. Another species, **C. ophicephali** was also reporeed by Pearse from Thailand in **Channa striatns**, but did not occur in fishes examined by the author.

Procamallanus sp.

Figure 7, 8

Definition : Spiruridea Diesing, 1861; Camallanidea Raiiet et Henri, 1915; **Procamallanus** Baylis, 1923.

Description : Small delicate worms, tapering slightly at anterior end. Cuticle with uniform conspicous transverse striations. Head bluntly rounded without lips, mouth opening round, directed anteriorly. Buccal capsule, subglobose, walls thickened posteriorly without obvious teeth, placgues, or stylet. Esophagus tapering slightly, broader at base, not clearly divided. Exeretory pore in middle third of esephagus. Nerve ring near junction of first and second third of esophagus. Intestine simple, without divertricula.

Female : (8 adult specimens)

Length 3.3 to 5.1 (4.3) mm. maximum width 77 to 121 (95.5) at midbody. Esophagus 260 to 410 (358.5) long and 33 to 88 (63.5) wide at base. Nerve ring 44 to 121 (91.7); average 25.6% from anterior end. Ovary in anterior half of body, 0.8 to 1.6 (1.21) mm. Uteri amphidelphic, extending to near anus posteriorly and to esophageal-intestinal junction anteriorly, filled with large globose eggs; gravid specimens with numerous developing embryos. Vulva 1.2 to 2.1 (1.7) mm. from anterior (average 37.6%). Tail conical, 66 to 121 (88) long; dorsoventral thickness at level of anus 39.6 to 44. Posterior extremity with two small lateral papillae; papillose at tip. Ratio of tail length to thickness at anus 1.6 : 1 to 2.7 : 1.

Male : (3 adult specimens)

Body length 2.0 to 2.1 (2.0) mm., maximum width 80 to 84 (82) at midbody. Esophagus 280 to 330 (305) long maximum width 33 to 39 (36) at base; slightly swollen at posterior end. Nerve ring 185 to 220 (200) from anterior extremity. Spicule single, slender, conspicuously straited; distal end pointed, and bent like a hook, 88 to 99 (93.5) long. Tail curved, 66 to 88 (80.6) long and 39.6 to 44 (42.4) wide at level of anus. Papillae paired, sixteen on either side: eleven preanal pedunculate, three postanal pedunculate, two adanal (one pedunculate and one sessile).

Posterior portion terminates in bursa - like structure divided into a dorsal and a pair of lateral lobes : each of the later contains short lateral lobule and a thick ventral lobule with a pair of papillary terminations on its inner surface; dorsal lobule thick more or less divided into a pair of latetal lobules and a papilla-like dorsal lobule all supported by broad caudal alae.

Host. Clarius macrocephalus
Anabas testudineous

Habitat : Stomach.

Discussion : The taxonomy of the genus is rather confused. Yamaguti lists more than 30 species, many of which are probably synonymous. Pearse (1933) reported finding 2 distinct species, **P. kerri** and **P. glossogobii** from **Glossogobius giuras** in Siam but did not describe them **Clarius batrachus** in both Ceylon and India is the host for **P. planoratus** (Annereaux, 1946) and in India for **P. clarius** (Ali, 1957) as well. Neither of the hosts recorded here has been previously reported. Until type maternal from the work of Pearse and Ali can be examined, no species diagnosis can be at attempted.

Cucullanus sp.

Figure 9

Difinition : Spiruridea Diesing, 1861; Cucullnidae Cobbold, 1864; Cucullaninae York of Maplestone, 1926; **Cucullanus** Mueller, 1777.

Description : Head with two lateral lips, each bearing two or three papillae. Esophagus muscular throughout with a club-shaped swelling posteriorly, and dilated anteriorly into a false buccal cavity.

Female ; (6 specimens)

Body length 8 to 11.2 (9.4) mm., 366 to 380 (374) wide. Tail conical. Vulva behind middle of body, 2.64 to 3.88 (320.4) mm. from posterior end. Ovary amphidelphic, oviparous; eggs thin-shelled.

Male : (4 Specimens) 6 to 9 (7.7) mm. in total length, maximum width, 260 to 340 (296) at midbody. Tail conical, bent ventrally, 245 to 250 (246) long. Precloacal sucker absent, caudal papillae present : 6 pairs of preanal, 2 pairs paraanal and 3 pairs postanal pappiliae. Spicules simple equal, distally alate, 154 to 156 (154.8) long; gubernaculum present.

Host. Puntius altus

Habitat. Intestine

Discussion : Species of Cucullanus have been widely reported from fishes of several different families but not from **Puntius altus**, therefore this is a new host record for this parasite. Yamaguti, 1941 reported the presence of another species, **Cucullanus cyprini**, from **Cryprinus carpio**, Japan; in the present investigation this parasite was not recovered from eight fishes of the same species. Yamaguti records 59 species of **Cucullanus**, many of which are

synonymous; it is evident that this genus is in need of revision.

Gnathostoma spinigerum larva

Host. Channa (Ophicephalus) striatus

Habitat. Small intestine.

Discussion: Only one specimen was recovered from the intestine of **Channa (O) striatus,** collected from Amphur Jom tong, Chiang Mai. The complete experimental cycle has been demonstrated by Prommas and Daengsvang (6).

ACANTHOCEPHALA

The Acanthocephalans are permanent parasites whose adult stages live in the intestine of vertebrates. Their name is derived from the spiny proboscis by mean of which they attach themselves firmly to the host's intestine. The sex are separate, and an intestine is lacking (7).

Two species of Acanthocephala were recovered during the survey.

Pallisentis gabaes (MacCallum, 1918)

Neoechinorhynchidea south well et Macfie 1952; Pallisentidae Van Cleave, 1928; Pallisentis Van Cleave, 1928.

Description : Body length 2.78 to 14. 79 (5.99) mm., trunk with a collar spines arranged in 6-14 closely set rings near anterior extremity. Posterior to this collar of spines is an unspined region which is followed by 20-25 widely spread rings of spines, remaining part devoid of spines. Procoscis short, 440 to 572 (470.4) long, cylindrical to globular, with four circles of 6-10 hooks each. Proboscis receptacle cylindrical to saccate, with single layer muscular wall. Testes cylindrical, contiguous. Cement gland long, cylindrical, syncytial, containing 6 to 9 nuclei.

Host. Channa striatus

Clarius batrachus

Habitat Intestine.

Discussion : Two hundred specimens were recover from both hosts. These specimens fitted closely to that of **P. gaboes** (Mac Callum, 1918). Other species of **Pallisentis** found in the **Channa sp.** are reported by Agarwal, 1958, in **C (Ophicephalus) punctatus;** India. Bhalerao, 1931, and Balis, 1933, in **C (O.) striatus**, India; Thapar, 1930, in **C (O.) marulius**, India.

Neochinorhynchus sp.

Definition : Neoechinorhychidea South well et Macfie, 1925; Neoechinorhychidae Van Clave, 1919; Neoechinorhynchinae Travassos, 1926, emend; **Neoechinorhynchus** Hamann, 1892.

Description : Body usually small, cylindrical, aspinose. Lacunar system consisting of medium dorsal and ventral longitudinal vessels and circular vessels which anatomose. Giant hypodemic nuclei almost always few (usually 4-5 dorselly and 1-2 ventrally). Proboscis short, globular; proboscis hooks in six spiral lows of three each; anterior hooks longer and stouter than other. Proboscis receptacle subcylindrical, raeher short, single layered.

Adult female : 7 to 11.2 (8.7) mm. long, bearing 6 spiral rows of 3 hooks each. The eggs are elipsoidal measuring 50 to 60 by 17 to 28.

Male : Body length 3.50 to 5.00 (4.4) mm., 350 to 540 (430)., maximum width. Testes contiguous tandem, followed by elongate syncytial cement gland, containing eight large nuclei; cement reservoir incorporated in posterior part of cement gland. Bursa bell-shaped.

Host. Pangasius lanaudii.

Habitat Large intestine.

Discussion : Yamaguti lists 44 species of the genus from all over the world, occuring in more than 50 species of fish representing many families. Of these species, **Neoechinorhynchus australe** Van Cleave, 1931, North America, and **N. hutchinsoni** Datla, 1936 India, are similar in size range. There is no previous record of **Neoechinorhynchus** from Thailand and none from the present host, **P. lanaudii.** The auther feels that there are insufficient grounds on this evidence alone to create a new species, pending examination of type material or the key to the species by Petrochenko, 1956.

Conclusions

Three species of helminths encountered in this survey are of potential public health importance. All are from popular food fishes in this area and have been well studied in the past : **Opisthorchis** metacercaria from **Puntius gonionotus ; Gnathostoma spinigerum** larva from **Spirometra sp.** from several hosts. The habitual consumption of raw or partially cooked fish will continue to be a source of infection despite widespread knowledge of the dangers involved.

Although 56% of the specimens in the survey harbored at least one helminth, and several were found to be infected with 2 or more species simultaneously, the parasites appeared to be well tolerated and their was no evidence of gross pathology noted. Infection of **Pangasius larnaudii**, on economically important food source with **Neaechinorhynchus sp.**, were generally very heavy and could potentially be a cause of morbidity in this host. The results of this survey indicate parasitism does not constitute a threat to fish population in the Mae Ping River or commercial fish ponds at this time.

Two species, previously described from Thailand by Pearse (1933) **Camallanus anabantis**, and **Procamallanus glossogobii** were recovered and are redescribed. Pearse also reported but did not describe on acanthocephalan worm, **Farzandia (Pallisentis) ophiooephali** from **Ophicephalus (Channa) striatus.** A similar species, **Pallisentis goboes** differing in the distribution of body spines. was recovered from

the same host, as well as from **Clarius batrochus**.

The intestinal fluke, **Oreintocreadium** is reported here for the first time in Thailand and **C. macrocephalus** is a new host record for the genus. Likewise, **Neoechinorhynchus**, though distributed throughout the world, has not been previously reported from Thailand, nor from **Pangasius**, its local host.

Both of the cestode parasites, **Caryophyllaeides** and **Bothriocephalus** are newly reported from Thailand and from new host. The former has not been here to fore recorded outside of Europe.

Lack of adequate, in country, library facilities together with the many toxonomic uncertainties among the genera encountered in this study, have made it extremity difficult to make reliaible identification at the species level. It is hoped future work will clarify these problems of taxonomy and further investigate the ecology and life history of these parasites.

Summary

During a survey on fresh - water fish in Chiang Mai Province, twelve species of parasites were reoccured, including 3 trematodes; 3 cestodes, 4 nematodes, 2 acanthocephala and one unknow metacercaria found in the flesh of **Anabas testudineous**.

Three are of importance as sources of human infection. **Opisthrochis** sp., **Sparganum** and **Gnathostoma spinigerum**.

TABLE 1

Family	Scientific Name	Common Name
Nandidae	Tilapia mosambica	Pla moh
	T. melanopleura	Pla moh kanglai
	T. nilotica	Pla nin
Anabantinae	Anabas testudineous	Pla moh Thai
Cyprinidae	Pantius viehoeveri	Pla cao
	P. genionotus	Pla cao
	P. altus	Pra tapientong
	Morulius chrysophekadion	Pla ka
	Cyclocheilichthys armatus	Pla tapien
Cyluridae	Wallagonia attu	Pla kao
Clariidae	Clarius batrachus	Pla duk dan
	C. Macrocephalus	Pla duk oi
Schilbeidae	Pangasius lanaudii	Pla ta poh
Bagridae	Mystus nemurus	Pla kott
Ophicepha lidae	Channa (Ophicephalus) striatus	Pla chon

Table 2 PARASITES RECORD FOR 53 INFECTED FISHES

Species	No. fish infected	Incidence %	Total no recovered	Range of number	Site of infection
Trematode					
Opisthorchis sp. metacercana	2	3.77	1000		fins, scales
Metacercaria type 2	1	1.88	3	3	meat
Oreintocreadium sp.	8	15.1	38	1–20	small intestine
Acanthostomatid sp.	4	7.54	21	1–10	small intestine
Nematoda					
Procamallanus sp.	4	7.54	47	2–20	stomach
Camallanus anabantis	3	5.4	5	1–3	stomach
Cucullanus sp.	1	1.88	8	8	intestine
Gnathostoma larva	1	1.88	1	1	small intestine
Cestoda					
Sparganum (larva of Spirometra)	10	18.8	59	4–12	intestine
Bothriocephalus sp.	1	1.88	1	1	intestine
Caryophyllaeides Nybelin	1	1.88	3	1	intestine
Acanthocephala					
Pallisentis gaboes	15	28.3	200	5–40	intestine
Neoechinorhynchus	3	5.4	70	20–35	stomach

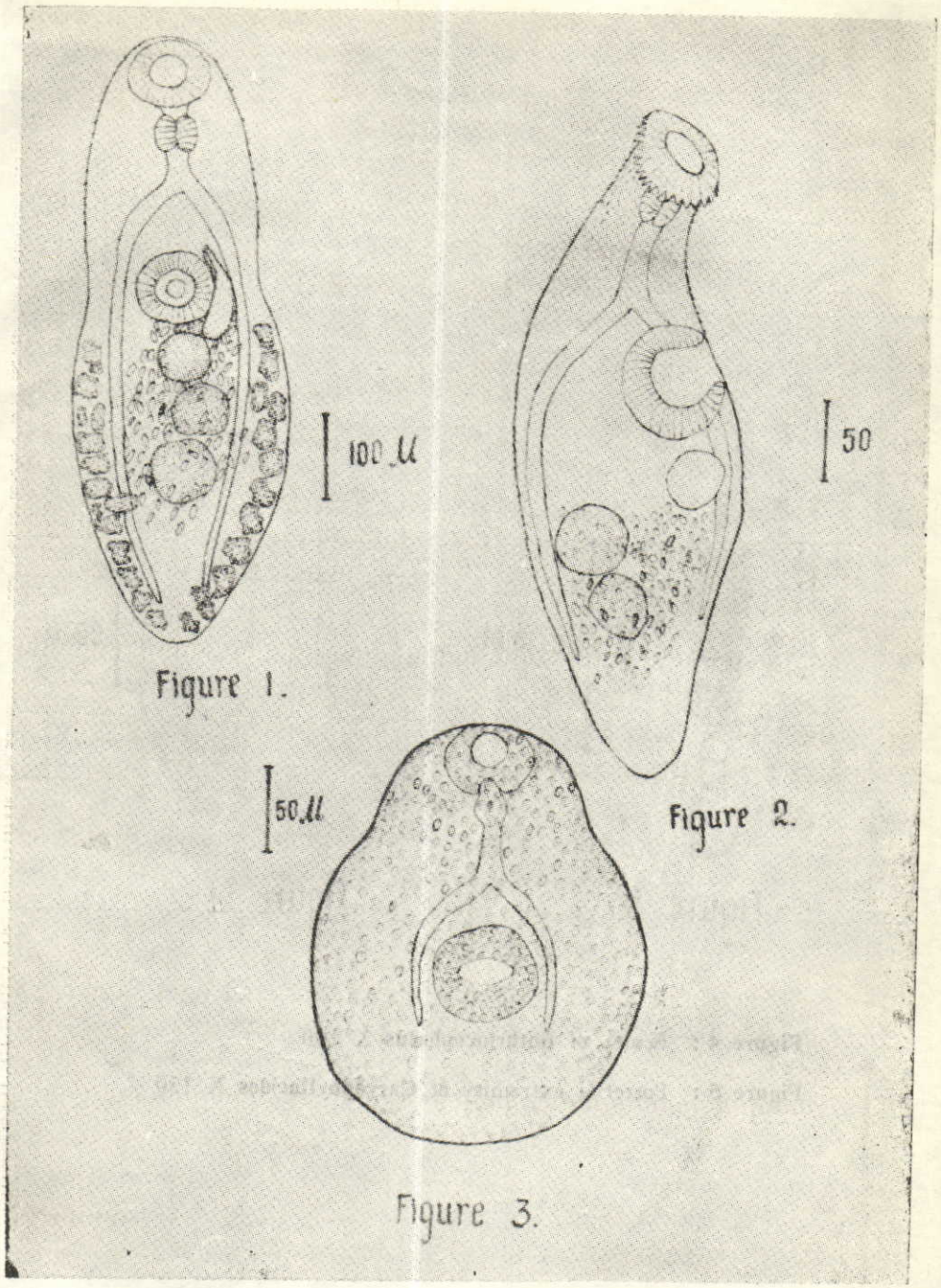

Figure 1 : Adult **Orientocreadium** from fixed and stained specimens X 50

Figure 2 : Adult **Acanthostomatid** from fixed and stained specimens X 300

Figure 3 : Unknown metacercaria in the flesh of Anabas testudineous X 300

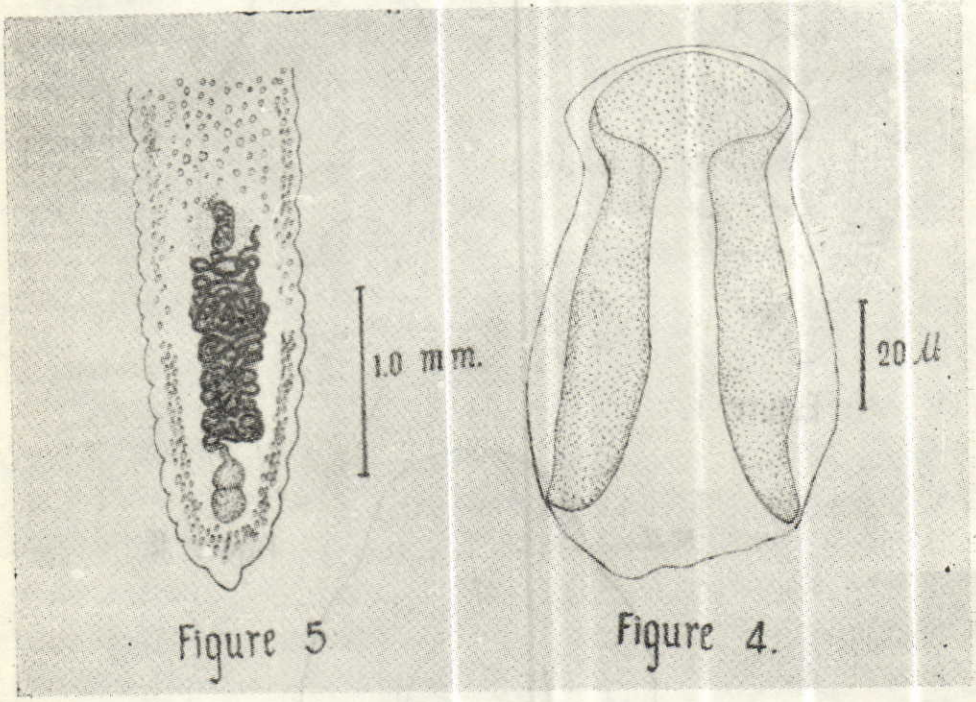

Figure 4 : Scolex of **Bothriocephalus** X 100

Figure 5 : Posterior extremity of **Caryophyllaeides** X 130

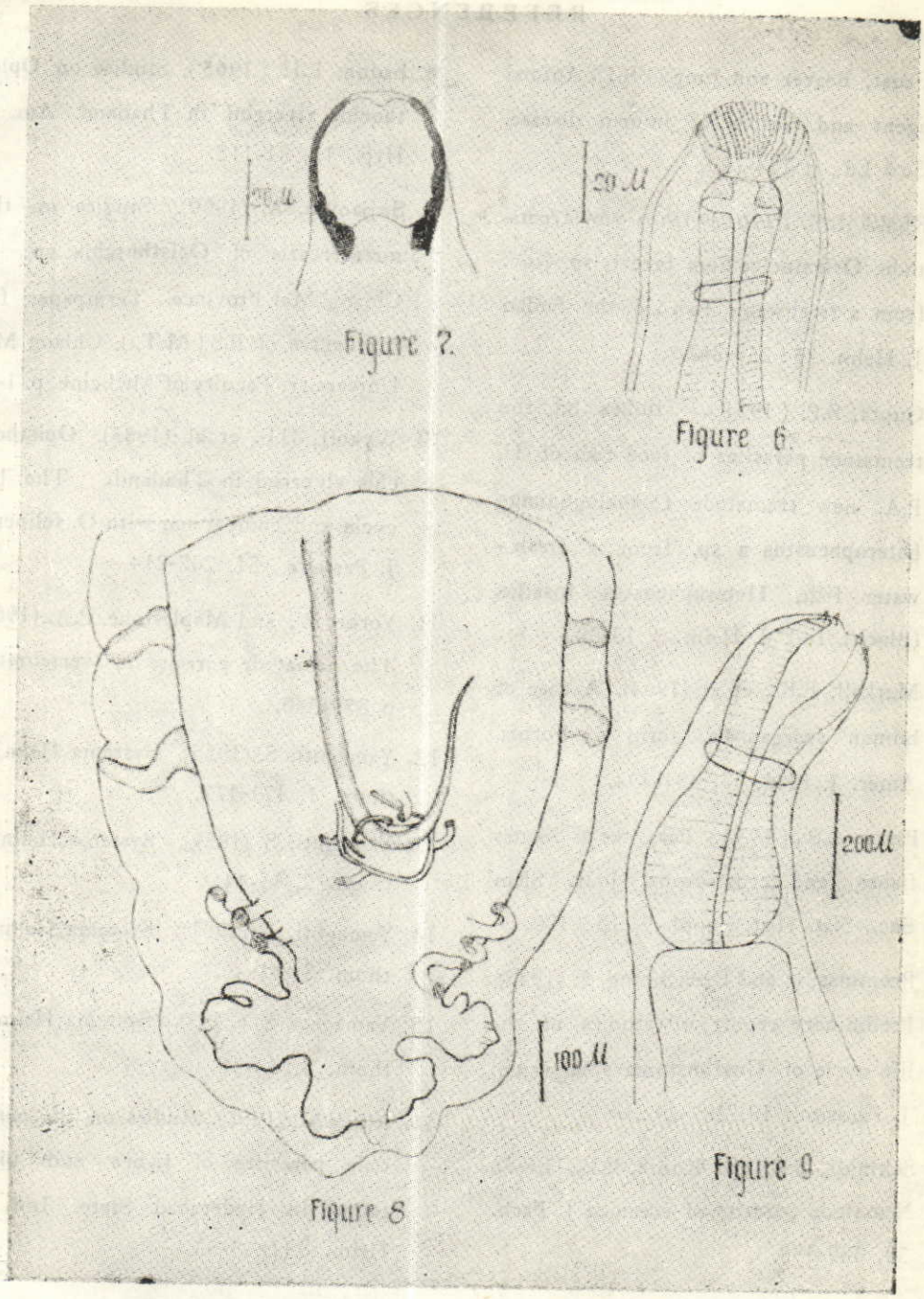

Figure 6 : Head of **Camallanus anabantis** X 55

Figure 7 : Head of **Procamallanus** X 120

Figure 8 : Posterior extremity **Procamallanus** X 450

Figure 9 : Head of **Cucullanus** X 50

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EFFECT OF COLLOCALIA MUCOID MEDIA ON TOXICITY AND NEURAMINIDASE ACTIVITY OF VIBRIO CHOLERAE*

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ABSTRACT

A study on the effect of **Collocalia** mucoid mucin media on **Vibrio cholerae** culture was carried out. The result showed that the organism grew well in this plain mucin media and mucin seemed to induce the enzyme neuraminidase production and increase the culture filtrate toxicity observed in rabbit skin test. It was suggested to use **Collocalia** mucoid or other mucins as **Vibrio cholerae** culture media for studying its virulence and pathogenicity.

INTRODUCTION

It has been reported that cholera diarrhea is caused by an exotoxin elaborated by cholera vibrios in the small intestine (5, 7, 9, 23). Cholera toxin has been isolated and characterized (8, 10). It is mixture of protein and enzymes (2, 6, 10, 15). An important enzyme, which is abundantly present in the **V. cholerae** enterotoxin is neuraminidase or recepter destroying enzyme (1, 11). Neuraminidase catalytically cleaves sialic acid or N-acetylneuraminic acid from various glycoproteins (12), neuraminyl lactose (13) and cell membrances (14, 27, 28). The surface of the intestinal mucosa is composed mainly of sialic acid-containing glycoproteins (18).

Vibrio cholerae can differently grow in various media such as alkali peptone water (22), desoxy cholate citrate medium (26), trypticase tellurite taurocholate peptone (20). Several media has been employed

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for the purpose to get a higher percentage of positive culture results (21). In this study, the suitable media for enhancement of the pathogenicity was tested, according to the fact that subcultures of this pathogenic organism **in vitro** causes a decrease or loss of their virulense and toxicity. Virulence may be restored by, **in vivo**, passage through mice. Using **in vitro** subcultures to retain the virulence of **V. cholerae** has not been reported. Mucin has been used to increase virulence of some organisms in animal passage by mixed with the organisms before injection (24). This similar effect should be studied in cholera.

One of the common mucin in Thailand is **Collocalia** mucoid. **Collocalia** mucoid is a mucin secreted from salivary glands of swiflet, living along the Pacific coast of Indian Ocean. It acts as a cementing substance in the bird's nest. It is commercially available, since Chinese and Asian people have been eating this bird's nest as a delicacy for their good health. Chemically, **Collocalia** mucoid from the edible bird nest is composed mainly of glycoprotein (16).

In this report, we have investigated the effect of using **Collocalia** mucoid as a culture media of **V. cholerae** on the toxicity and the neuraminidase activity of the organism.

MATERIALS & METHODS

Collocalia mucoid : This was prepared from edible bird nest bought from a local market. The mucin was extraced from the powdered bird nest with warm water as described by Howe et al. (17).

Vibrio cholerae : The strain El Tor Inaba Chachoengsao which gives the lowest neuraminidase activity in all six stains (kindly recieved from SEATO Laboratories, Bangkok) was used for this study. The freeze-dried organism was suspended in 1% alkali peptone broth pH 8.4, incubated at 37° C for six hours, then streaked on blood agar plate to obtained isolated colony of **V. cholerae**.

Tryptone broth : Bacto tryptone was purchased from Difco Laboratories, Detroit 1, Michigan, U.SA.

Three kinds of Media were prepared :

1. **Tryptone Broth**. 2% Bacto tryptone in saline (0.5% NaCl) was adjusted to pH 7.5 with 4 M NaOH. The organism from isolated colony on blood agar plate was suspended in distilled water to reach Mac Farland No. 2.0 turbidity. The 0.05 ml. of cell suspension was inoculated into 20 ml. tryptone broth, incubated at 30° C for 18 hours. The culture was centrifuged to remove the cells, and the supernatant fluid was filtered through the millipore filter membrane by using filtering centrifuge tube, This filtrate was streaked on

blood agar plate in order to test the sterility. The culture filtrate, as control in this study, was stored at 4° C for determining the neuraminidase and toxicity.

.2. **Mucin in Tryptone** broth. This media was made by adding 0.2 gm. of **Collocalia** mucoid in 100 ml. tryptone broth prepared as above. The same strain of organism was subcultured into 5 ml. of this media for 10 times, at 24-hour intervals. The treated organism was streaked on blood agar plate to get pure isolated colony. The same turbidity and volume of cell suspension was used for culturing in tryptone broth to obtain culture filtrate as above. The culture was brought for determination of neuraminidase activity.

3. **Mucin Media** No tryptone was used in this media. The media contained 2.7 % **Collocalia** mucoid in 0.5 % NaCl. The pH of 7.5 was also adjusted. The same strain of **V. cholerae** was treated in 5 ml. of this media with seven subcultures at 24-hour intervals. Finally, the treated organism was cultured in tryptone broth after isolating on blood agar plate, to obtain a culture filtrate as described before. The culture filtrate was taken for determination of neuraminidase activity and toxicity.

Detection of Toxicity :

By using skin test of rabbit, the toxicity of the culture filtrate was indicated by the edema or swelling of the animal skin. An aliquot of 0.2 ml. of the culture filtrate was injected intradermally into rabbit back skin after hair removal. A 5-minute heated culture filtrate was also run as a control. Any visual change of the injected skin was recorded.

Determination of Neuraminidase Activity :

Disappearance of bound sialic acid in substrate was used as the enzymatic activity of neuraminidase. The action of neuraminidase is to hydrolyse bound sialic acid into free molecules. Orosomucoid or serum L-acid glycoprotein was used as the substrate. The substrate was dissolved in acetate buffer (1.25 gm. orosomucoid in 100 ml., 0.075 M acetate buffer containing 0.3% $CaCl_2$), pre-incubated at 37°C, then the substrate mixture and the cholera culture filtrate were mixed together with a volume ratio of 4 to 1 respectively.

As soon as the cholera culture filtrate was well mixed with the substrate mixture, 0.2 ml. aliquots of the incubation mixture were withdrawn at 0, 5, 10, 20, 30, 60 and 90 minutes. The aliquots were heated at 100°C in boiling water for 3 minutes to stop enzymatic action. Bound sialic acid content in the incubation mixture was analysed by periodate - resorcinol method (19). A decrease of bound sailic acid within 20 minutes of incubation time indicated the initial activity of the enzyme.

RESULTS

Rabbit Skin Test for Toxicity. The result is summarized in **Table 1.** The culture filtrate obtained from the original organism gave no sign of swelling on the rabbit skin within 24 hours. It showed a little swelling in 48 hours, and on the third day the local lesion was observed.

In the case of mucin media presubcultures, the organism produced the culture filtrate which showed higher toxicity than that from the original untrated one. For it gave the observation of skin swelling within 24 hours and the local lesion occured at 48 hours after injection.

TABLE 1

Toxicity of Vibrio cholerae culture filtrate from different culture media by rabbit skin test.

Culture media	Swelling observation in	
	24 hrs.	48 hrs.
1. Tryptone broth (as control)	−	+
2. Mucin presubcultures	+	++

Neuraminidase Activity Table 2 shows the activity of neuraminidase in the culture filtrates using orosomucoid as a substrate. The activity of enzymes is expressed as Units per ml. culture filtrate. One Unit of enzyme is expressed as the enzyme that can liberate one ugm. of sialic acid from orosomucoid at pH 5.5 in 15 minutes at 37°C (25). Each initial velocity from the plot shown in Figure 1. is used in calculation.

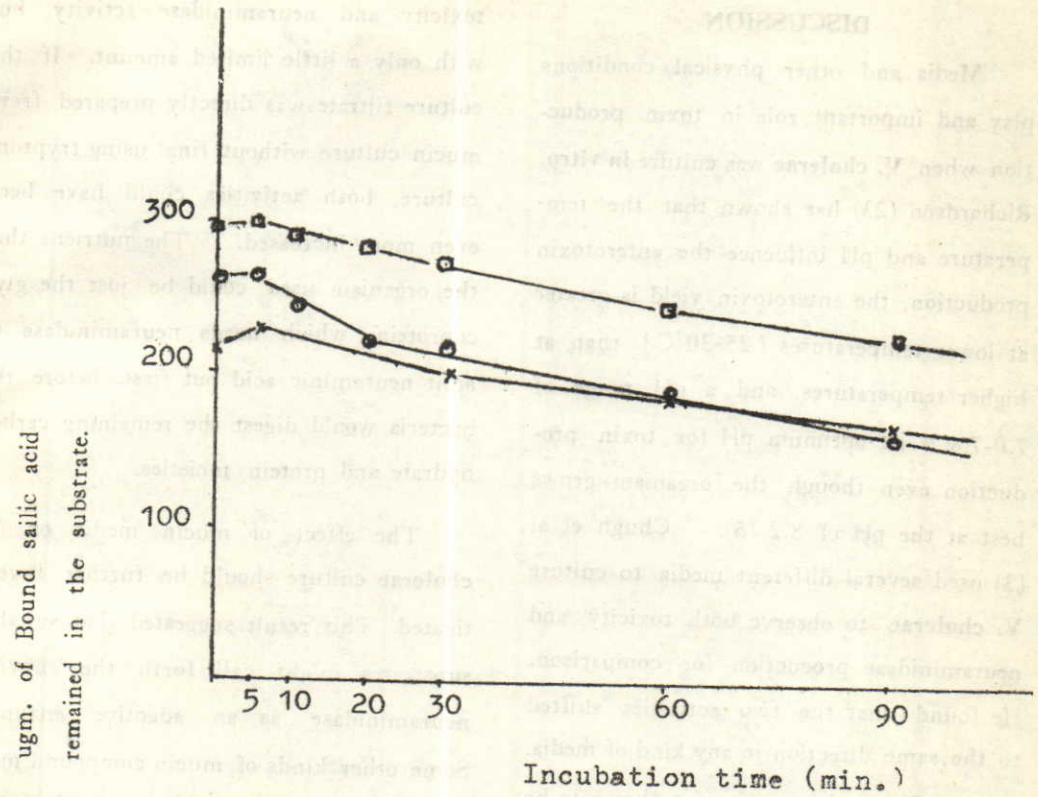

Fig. 1. Enzymatic activity of neuraminidase in **V. cholerae** culture filtrates obtained from different types of subculture media; ×—×, control, □—□, mucin tryptone subculture media, 0—0, mucin subculture media.

TABLE 2

Neuraminidase activity of **V. cholerae** culture filtrates obtained from different culture media. Numbers in parentheses are numbers of subcultures.

Culture media in subculture	Enzymatic activity (Units/ml,)
Tryptone broth (0)	75
2% Mucin in tryptone broth (10)	75
Mucin only (7)	200

DISCUSSION

Media and other physical conditions play and important role in toxin production when **V. cholerae** was culture **in vitro.** Richardson (23) has shown that the temperature and pH influence the enterotoxin production, the enterotoxin yield is greater at lower temperatures (25-30°C) than at higher temperatures, and a pH range of 7.0-7.8 is an optimum pH for toxin production even though the organism grows best at the pH of 8.2 (5). Chugh et al (3) used several different media to culture **V. cholerae** to observe both toxicity and neuraminidase procuction for comparison. He found that the two activities shifted to the same direction in any kind of media.

In this study, mucin was shown to be a very simple media for **Vibrio cholerae.** No other integradient was needed to make it enriched. This media was made to imitate the nature of intestinal epithelium which contains mucins or glycoproteins (18). It was observed that even the organism was cultured in plain mucin solution, it was able to grow quite well. The result showed that after the organism was incubated several times in mucin media it tended to give a higher degree of both toxicity and neuraminidase activity, but with only a little limited amount. If the culture filtrate was directly prepared from mucin culture without final using tryptone culture, both activities could have been even more increased. The nutrient that the organism used could be just the glycoprotein, which needs neuraminidase to split neuraminic acid out first, before the bacteria would digest the remaining carbohydrate and protein moieties.

The effect of mucin media on **V. cholerae** culture should be further investigated. This result suggested that suitable substrates might call forth the enzyme neuraminidase as an adaptive enzyme. Some other kinds of mucin compound may be better or perhaps some other suitable substance and some physical conditions are needed for more production of neuraminidase and toxin. These conditions should be similar to that in the intestine of the cholerae patient. Finally this might be an economical means to enhance the vilurence or induce the enzyme production of **Vibrio cholerae** by **in vitro** subcultures, instead of using **in vivo** orderliness as generally employed by microbiologist.

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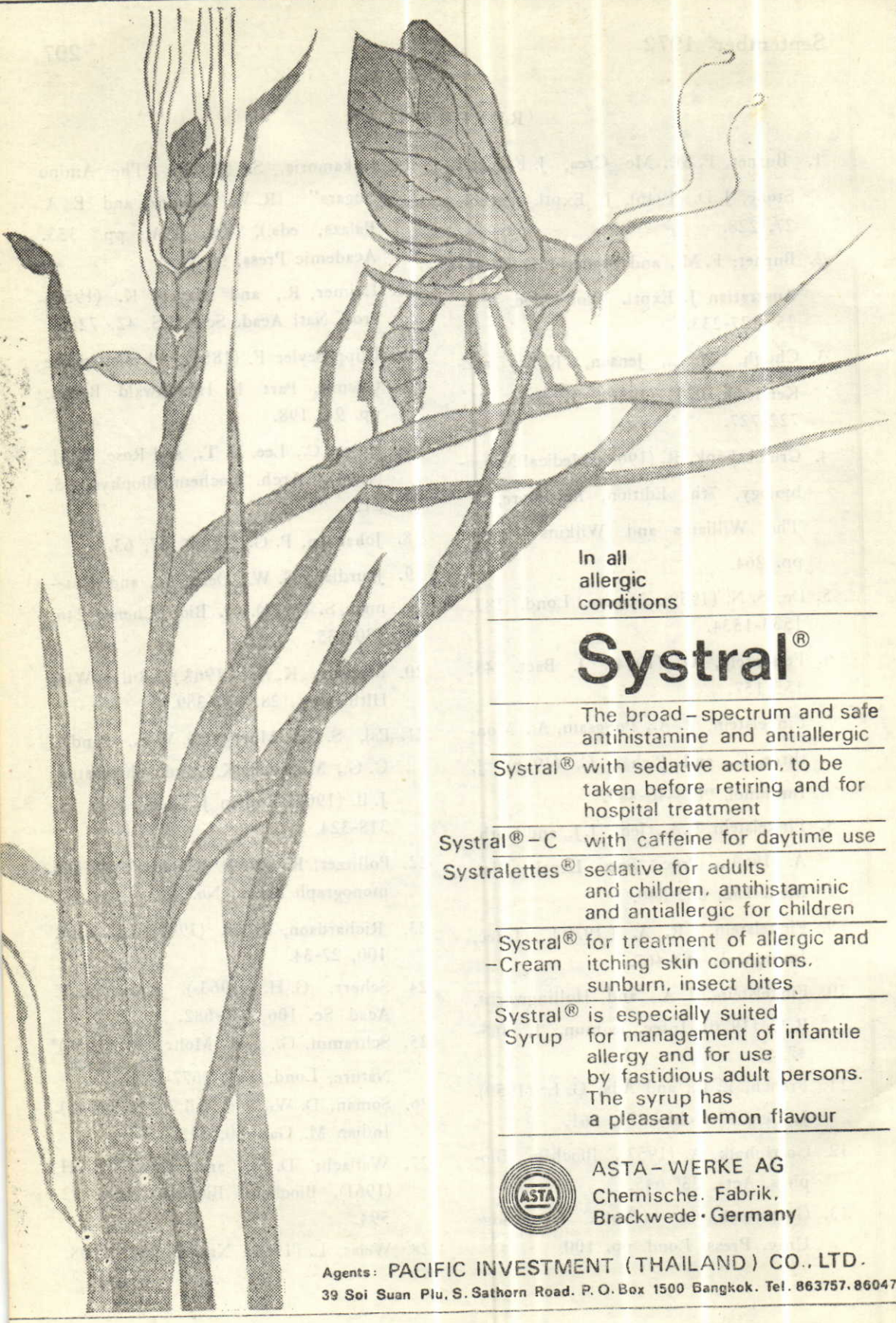

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FOUR ANTIBIOTICS SENSITIVITY TO MICROORGANISMS IN LOCAL WOUND INFECTION

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Abstract

The susceptibility to antibiotics of microorganisms isolated from various wounds and burns infections of the patients in Nakorn Chiang Mai Hospital were investigated. The antibiotics sensitivities test was performed by serial tube dilution method to four antibiotics; Soframycin (Framycetin sulphate), Garamycin (Gentamycin), Terramycin (Tetracycline), and Colimicin (Colistin methane sodium). From 79 specimens, the isolated organisms were **Staphylococcus aureus** (34 strains), **Escherichia coli** (10 strains), **Proteus mirabilis** (8 strains), Klebsiella (7 strains), Pseudomonas aeruginosa (7 strains), and Enterobacter (4 strains) respectively. The results indicated that 85% of the isolated strains of Pseudomonas aeruginosa were sensitive to garamycin and colistin, and 25% were shown to be resistant to Soframycin. In case of **Staphylococcus aereus**, 97.5 %, 91.4 %, 61.8 % and 61.8 % were sensitive to garamycin, soframycin, terramycin and colistin respectively. Almost all strains of **Proteus mirabilis** were resistant to all antibiotics. All strains of **Klebsiella** were sensitive to garamycin and only 42.8 % to the other three antibiotics. **Enterobacter** were found sensitive to garamycin, soframycin, colistin and terramycin at the percentage of 75, 50 and 25 respectively.

Introduction

Antibiotics have been successfully employed in the treament of infections disease. The feasibility of using antibacterial substances **in vivo** as chemotherapeutic agents depends primarily upon the specific action of such substances, regarding to **selective action** on the microorganisms

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without significant harmful effects on the host (3). The microorganisms differ markedly in resistance and sensitivity to the various antibiotics, not only between genera and species but also between strains of the same species. They may produce enzyme to neutralize antibiotic such as Penicillinase in **Staphylococcus aureus** which can inactivate penicilline (2). Some of them have mutate into resistant strains to some antibiotics. However, it is not neccessary to test the sensitivity of some organisms such as **Diplococcus pneumonia, Staptococcus pyogenes, Neisseria meningitidis, and Neisseria gonorrhea** (12). Because they are always sensitive to penicillin.

The wound infection is usually caused by contamination of serrounding bacteria which includes bacteria from skin, clothes and dust. (4) Staphylococcus aureus is the most frequently found in this case, the other include **Streptococcus pyogenes** (Lancefield's group A), Spore forming bacilli, (Aerobic and Anaerobic) **Enterococci**, hemolytic and non-hemolytic streptococci, **Diphtheroids**, Gram negative intestinal bacilli, **Pseudomonase aeruginosa, Proteus Pasturella septica** (5).

In case of post operation the inflamation of wound is mostly caused by contaminative **Staphylococcus aureus** from air, nasal cavity of staff hospital, or from skin, clothes, and blankets of the patients (6).

The patients with burn infection are frequently found infected with **Pseudo monas, Staphylococcus, Sterptococcus phyogenes.** Other organisms less frequently found in burn infections are **Proteus, Klebsiella** and **Enterobacter.** (7)

Baber et al (2) performed an experiment of Bacterial resistance to antibiotic, finding that the number resistance strains of microorganisms to antibiotic will increase with the period of that antibiotic used. It is then important that susceptibility of pathogenic organisms isolated from patients be done to guide to the selection of proper antibiotic for treatment.

Material and Method

1. Specimens and organisms

The specimens were swab from inflamation area both superficial and deep, from operative incisions and burns from patients at O. P. D. of Nakorn Chiang Mai Hospital. A fresh swab was inoculated into the following media; Blood agar, Mc. Conkey agar, EMB and Thioglycolate broth (for anaerobic organisms). The transport media was tryptricase soy broth with sterile cotton swab in 10 ml. test tube. The isolated microorganisms from specimen were identified by using biochemical test. (Bailey & Scott (1)). The pure cultures were preserved in

trypticase soy agar, and stored at room temperature or refrigerator.

II. Antimicrobial agents

Four antibiotics were used in the experiment :

1. Soframycin 2. Colimycin 3. Terramycin and 4. Garamycin. These antibiotic were dissolved in sterile distilled water to make concentration being 2000 mcg or unit / ml and divided them to 4 ml. in each bottle store in deep freeze for stock solution. Solution was thawed and it was diluted to 200 mcg. or unit/ml. for using in the test, and the remaining stock solution from each test was discarded.

III. Susceptibility testing method

Stock solutions of antimicrobial agents were thawed and diluted with sterile distilled water, the final concentration being 200 mcg or unit/ml. The serial dilution of antimicrobial was done using trypticase soy broth as diluent to make concentration of 100, 50, 25, 12.5, 6.25. 3.125, 1.562, 0.781 and 0.36 mcg or unit / ml., the final volume being 0.5 ml. Then 0.5 ml of 1 : 1000 dilution of overnight culture of organisms in trypticase soy broth was added to each antimicrobial dilution. Incubate 37°C overnight and read MIC (Minimal Inhibitory Concentration).

Results

The total of 79 strains of various organisms were isolated from wound infections as shown in **table 1. Staphylococcus aureus** was the predominant microorganisms. The Minimal Inhibitory Concentration of each organisms to four antibiotics were shown in **table 2.** with their MIC standard of antibiotics shown in **table 3.** The percentage of sensitive, intermidiate, and resistant of organisms to antibiotics are shown in **table 4.** Most strains of **Staphylococcus aureus**, **Pseudomonas aeruginosa**, **Proteus mirabilis**, **Klebsiella** and **Enterobacter** are sensitive to garamycin. Most of the strains of these microorganisms are resist to terramycin.

Discussion

The determination of susceptibility of pathogenic organisms to antibiotic is very important in the selection of antibiotic for therapy. The organisms are different in their susceptibilities to antibiotics. There are many methods determine susceptibility of organisms to drugs, but the quantitative accuracy one is serail tube dilution method. Many factors such as; pH of media, incubation time, inoculum size, and stability of drugs (8), may influence in the detection of minimal inhibitory concentration of each drug.

From many report (9, 10) we found that the strains of **Staphylococcus aureus** isolated from hospitals resist penicillin.

These incidences have been attributed to the fact that large-scale use of penicillin in the hospital has wiped out the penicillin sensitive **Staphyloccus aureus;** thus remaining only resistant variants (usually producing penicillinase). A similar situation has accured to gram-negative organisms, especially hospital strains. The excessive use of drug lead to suppression of drug susceptible microorganisms and favors the survival of drug resistant ones such as; **Aerobacter, Proteus, Pseudomonas** species.

From the results it is expected that garamycin and soframycin are excellent for the treatment of local wound infection. Although soframycin, only has intermidiate sensitivity effect on strains of **Pseudomonas aeruginosa.** Salcida and coworker(30) reported, however soframycin was the best drug for the treatment of corneal infection cause by **Pseudomonas aeruginosa** without any harm to normal ocular tissue.

Most of strains of tested organisms were not sensitive to terramycin. These might be due to the wide spread use of terramycin resulting in elimination of tetraccycline sensitive organisms.

In addition soframycin and garamycin are active against gram-positive cocci and gram-negative bacteria including penicillin resistance Staphylococci. It is therefore useful in the treatment of mixed organisms in wound infection. Both soframycin and garamycin are relatively new antibiotic, so the percentage resisting organisms is not very high.

Table 1

79 Isolated Organisms from various wounds, Abscesses and Burns.

Isolated Microorganisms	No. of Isolated
Staphylococcus aureus	34
Escherichia coli	10
Proteus mirabilis	8
Pseudomonas aeruginosa	7
Klebsiella	7
Enterobacter	4
B-Hemolytic Streptococci	6
Gram-negative Anaerobic Bacilli	3
Total organisms	79

Table 2

Minimal Inhibitory Concentration (MIC) of 79 strains of Isolated organisms to four antibitics.

Organisms	Garamycin M I C (mcg/ml)	Colistine M I C (mcg/ml)	Terramycin M I C (mcg/ml)	Soframycin M I C (mcg/ml)
34 Strains of Staphylococcus asreus	0.39/22*	100/4	0.39/19	0.39/8
	0.78/8	> 100/30	0.78/12	0.78/9
	1.56/3		3.125/2	1.56/6
	3.125/1		12.5/1	3.125/8
			25.0/2	6.25/2
			50.0/7	25.0/1
			> 100/1	
10 Strains of Escherichia Coli	1.56/1	6.25/1	1.56/3	6.25/1
	3.125/5	12 5/1	12.5/1	12.5/3
	6.25/2	25.0/1	> 100/6	25.0/5
	12.5/1	50/1		50.0/1
		100/2		
		> 100/1		
8 Strains of Proteus mirabilis	0.39/1	> 100/8	3.125/1	3.125/1
	1.56/1		12.5/1	6.25/1
	6.25/3		100/5	25.0/5
	12.5/3		> 100/1	50.0/1
7 Strains of Pseudomonas aeruginosa	0.39/1	25.0/2	1.56/1	6.25/3
	0.78/3	50.0/2	3.125/2	12.5/1
	1 56/3	100/2	6.25/3	25.0/2
	100/1	> 100/1	100/1	> 100/1
7 Strains of Klebsiella	0.39/2	25/3	0.78/3	3.125/3
	0.78/3	> 100/4	3.125/1	6.25/3
	1.56/2		12.5/1	100/1
			50.0/1	
			> 100/1	

(continue)

Organisms	Garamycin M I C (mcg/ml)	Colistin M I C (mcg/ml)	Terramycin M I C (mcg/ml)	Soframycin M I C (mcg/ml)
4 Strains of Enterobacter	0.78/1	50.0/1	0.78/1	3.125/2
	1.56/1	> 100/3	6.25/1	6.25/1
	50.0/1		25.0/1	100/1
			100/1	

* 0.39/22 = 22 strain of Staphylococcus aureus has MIC = 0.39 mcg/ml

Table 3

Showing the standard limit of Minimal Inhibitory Concentration for "Sensitive", "Intermediate", and "Resistant" strains.

Antibiotics	Sensitive (mcg/ml)	Intermediate (mcg/ml)	Resistant (mcg/ml)
Gentamycin	≤ 2.5	> 2.5–10	> 10
Colistin	≤ 100 u/ml	> 100–500 u/ml	> 500 u/ml
Tetracyclin	≤ 2.5	> 2.5–5.0	> 5.0
Streptomycin*	≤ 5.5	> 5.0–25	> 25

*Streptomycin have Chemical Semilar to Soframycin.

Table 4

Antibiotic Susceptibility of Pathogenic organisms Isolated from various wounds and burns.

Percentage of sensitive, Intermediate, and Resistant.

Isolated org.	No. of strains	Garamycin mcg/ml			Colistin unit/ml			Terramycin mcg/ml			Soframycin mcg/ml		
		% S	% I	% R	% S	% I	% R	%S	% I	% R	% S	% I	% R
Staphylococcus aureus	34	97.5	2.5	0	11.8	88.2	0	61.8	11.8	26.4	94.1	5.9	0
Escherichia coli	10	10	80	10	50	50	0	30	0	70	0	90	10
Proteus mirabilis	8	25	37.5	37.5	0	100	0	0	12.5	87.5	12.5	50	37.5
Pseudomonas aeruginosa	7	85.8	0	14.2	85.8	14.2	0	14.3	42.8	42.8	0	85.8	14.2
Klebsiella	7	100	0	0	42.8	57.2	0	42.8	14.3	42.8	42.8	42.8	14.3
Enterobacter	4	75	0	25	25	75	0	25	0	75	50	25	25

S = Sensitive

I = Intermediate

R = Resistant

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ย่อ และ รีวิวเอกสาร

Erythrocyte Content of Free Protoporphyrin in Thalassemic Syndrome

C. Lyberatos, G. Chalevelakis, A. Platis, N. Stathakis, A. Panani and C. Gardikas. Acta Hematologica Vol. 47, No 3, 1972 (P. 164—167)

ในการ ทด ลอง หา ค่า Erythrocyte protoporphyrin (EP) ในคนปกติและคนไข้ที่เป็น Thalassemia

ผู้รายงานได้ศึกษาโดยการทด ลอง ใน คน ปกติ 20 คน ที่ไม่มีอาการของโรคเลือดเลย คือมีระดับ Hemoglobin 14 gm%, serum Iron ปกติ, Saturation Total Iron-binding Capacity มากกว่า 16% ได้ค่า EP 25.6 $\pm$ 9.54 ug/100% ml erythocyte range 11–38 ug/100 ml E.

ในคนไข้ hetorozygous B–thal. (minor) 31 คนได้ค่า EP 70.4 $\pm$ 22.19 ug/100 ml erythocyte rang 44–108 ug/100 ml E.

ในคนไข้ homozygous B – thal (major) 20 คนได้ค่า EP = 83.3 $\pm$ 23, 42 ug/100 ml erythrocyte rang 55–156 ug/100 ml E

จากการทดลองปรากฏว่า ค่าที่ ได้ แตก ต่าง กันเห็นได้ชัด คือมีค่าสูงในคนไข้ thal. assemia.

อัญชลี กิตติชนม์ธวัช
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Phagocytic Activity of Multiple Myeloma Cells

Patricia Ferguson and Paul L. Woff. Laboratory Medicine. 3 : 32 - 33 (1972)

พบคนไข้ Multiple Myeloma ราย หนึ่ง เป็นชายวัย 75 ปี ซึ่งป่วยหาหมอด้วย อาการของ external dyspnea และ mild substernal discomfort ย่อนเพลีย เคย มีประวัติเป็น Arteriosclerotic Heart Disease มาก่อน การตรวจทางร่างกายไม่ให้ ลักษณะที่สำคัญอะไร ไม่มีตับม้ามโตและอาการของการปวดกระดูก ผลของ C B C มี white blood cell count 6,000, hemoglobin 8.6 และ blood indicies ปกติ จากการตรวจทางเคมีพบว่า albumin เพียง 2.6 gm. globulin สูงถึง 8.6 gm. และพบ monoclonal gammapathy.

ที่น่าสังเกตอีกอย่างหนึ่งก็คือ ไม่ปรากฏมี

Bence–Jones Protein ในปัสสาวะ และจากการตรวจทาง microscopy พบแต่ hyalin casts จำนวนเล็กน้อยเท่านั้น

จุดของการตรวจพบที่สำคัญอยู่ที่กระดูกพบว่ามี bone demineralization. Bone marrow smear เต็มไปด้วย plasmoblast และ 1% ในพวกนี้พบ phagocytosis ของ platelets, red blood cells และ lymphocytes โดยเฉพาะ platelets และ rbe จะถูกกินมากที่สุด การเจริญของเม็ดเลือดสาย Erythroid, Myloid และ Megakaryocytes มีน้อยลง

โดยปกติแล้ว Myeloma cells จะไม่แสดงปรากฏการณ์ของ phagocytic activity ต่อ rbc, plateletes หรือ lymphocytes. ก่อนหน้านี้ (ปี 1953) มีรายงานอันหนึ่ง พบ Plasmacytic phagocytosis ของ rbc ใน periferal blood และ bone marrow smeas ของคนไข้ Plasma Cell Leukemia. เคยมีการทดลองฉีด carbon particle เข้าในร่างกายของคนไข้ Multiple Myeloma พบว่า Myeloma cells ไม่กิน foreign material คือไม่มี phagocytic activity.

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Comparison of A Macro & Micro Method for Determining Red Blood Cell Fragility. Betty Day Quick, Amarican Jounal of Medical Technology 38, 163-170 1972.

Micro Method ที่ใช้ในการหา Red biood cell frsgility นี้เป็นวิธีของ Mc-Cormick. สำหรับ Macro Method ใช้วิธีของ Lovinson MacFate. โดยวิธีของ Mc Cormick นั้น เตรียม buffered saline (NaCl 180.0 gm., $NaHPO_4$ 27.31 gm., NaH_2PO_4–H_2O 4.86 gm. ละลายในน้ำกลั่น 2 ลิตร) เป็น stock solution.

จาก stock solution นำมามา dilute 2 : 10 จะได้ 1% NaCl เพื่อใช้ในการทำ 14 serial dilutions. มี concentration ของ NaCl ตั้งแต่ 0.25% จนถึง 0.90% แต่ละ dilution มี concentration ต่างกัน 0.05% Working solutions เหล่านี้เก็บไว้ที่ตู้เย็นเพื่อป้องกัน contamination.

ใช้ Hematocrit capillary tube ทำเครื่องหมายที่ 40 mm. และ 50 mm. จากปลาย 14 tubes. ใส่ working solutions ลงให้ถึงขีด 40 mm. แล้วต่อด้วยเลือดจนถึงขีด 50 mm. Seal ปลายอีกด้านหนึ่งแล้วคว่ำลงตั้ง เลือดจะผ่านน้ำเกลือลงไปข้างล่างอย่างช้า ๆ เป็นการ mix ไปในตัว ตั้งไว้

30 นาที Centrifuge 3 นาที แล้วอ่านผลของ hemolysis

จากการเปรียบเทียบผลของการทำ micro method นี้กับวิธีของ Levinson MacFate ปรากฏว่าให้ผลถูกต้องใกล้เคียงกันมาก กล่าวคือในคนปกติ โดยค่าเฉลี่ยเลือดจะเริ่มเกิด hemolysis ใน tube 0.40 % ± 0.20 % ใน micro method และ 0.45% 0.45 % ± 0.02% ใน macro method. คนไข้ 11 คนที่เป็น Lead poisoning จะเริ่มมี hemolysis ใน tube 0.30 % สำหรับ micro method และ 0.35 % สำหรับ macro method.

วิธี micro Method ของการหา rbc fragility นี้มีผลดีมากกว่า micro method หลายประการ สิ้นเปลืองเวลาน้อยกว่า ใช้เลือดจำนวนน้อยอันเป็นข้อดีที่จะใช้กับเด็ก ซึ่งทำ venipunture ยาก อุปกรณ์เครื่องใช้กระทัดรัด ทำได้ง่าย และสิ้นเปลืองน้อยและผลที่ออกมาถูกต้องใกล้เคียงกับ macromethod.

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ข่าว

สมรส

เทคนิคการแพทย์ ที่เข้าสู่พิธีมงคลสมรสในระยะนี้ คือ

คุณวัลย์ดา อาศนะเสน เทคนิคการแพทย์ กรุงเทพฯ รุ่นที่ ๑๑ (บัณฑิตเทคนิคการแพทย์ เชียงใหม่ รุ่น ๓) แห่ง รพ. จุฬาลงกรณ์ สมรสกับ คุณจิตรบูรณ์ ณ สโมสรกองทัพบก สี่เสา เทเวศน์ เมื่อวันที่ ๒๔ สิงหาคม ๒๕๑๕

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ทุนอุดหนุนการศึกษา

น.ส. แจ่มใส ผ่ายอินทร์ นักศึกษาเทคนิคการแพทย์ปี ๒ ได้รับทุนการศึกษา ของคณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่ ประจำปี ๒๕๑๕ โดยได้รับเป็นทุนเดือนละ ๓๐๐ บาท เป็นเวลา ๑๐ เดือน

ทุนอบรมต่างประเทศ

อาจารย์ กิจจา สุจิต เทคนิคการแพทย์ เชียงใหม่ รุ่นที่ ๒ แห่งภาควิชารังสีวิทยา คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่ ได้รับทุนโคลัมโบ ให้ไปฝึกอบรมด้านรังสีวิทยา ในแขนง Radiology in physics ณ เมืองบอมเบย์ ประเทศอินเดีย มีกำหนด ๑ ปี และจะออกเดินทางในราวต้นเดือนตุลาคม ๒๕๑๕ นี้

การปฐมนิเทศก์อาจารย์ใหม่ และอภิปรายกลุ่ม

มหาวิทยาลัยเชียงใหม่ จะจัดให้มีการปฐมนิเทศก์อาจารย์ใหม่ และอภิปรายกลุ่ม ในวันที่ ๒๗–๓๐ ตุลาคม ๒๕๑๕ ณ หอประชุมโรงเรียนบ้านสันโค้ง อ. เมือง จังหวัดเชียงราย โดยผู้จะเข้าร่วมการปฐมนิเทศก์นั้น ต้องเป็นข้าราชการตำแหน่งอาจารย์ ซึ่งบรรจุเข้ารับราชการ หรือโอนมาบรรจุเข้ารับราชการในมหาวิทยาลัยเชียงใหม่ ระหว่างวันที่ ๑ กรกฎาคม ๒๕๑๓ ถึงวันที่ ๓๐ กันยายน ๒๕๑๕

อนึ่ง มหาวิทยาลัยเชียงใหม่เคยจัดสัมนาอาจารย์ครั้งแรกที่เขื่อนภูมิพล ระหว่างวันที่ ๒–๕ กรกฎาคม ๒๕๑๓ ซึ่งผลของการสมนาเป็นที่น่าพอใจ และเป็นประโยชน์แก่ราชการของมหาวิทยาลัยเชียงใหม่เป็นอย่างมาก.

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