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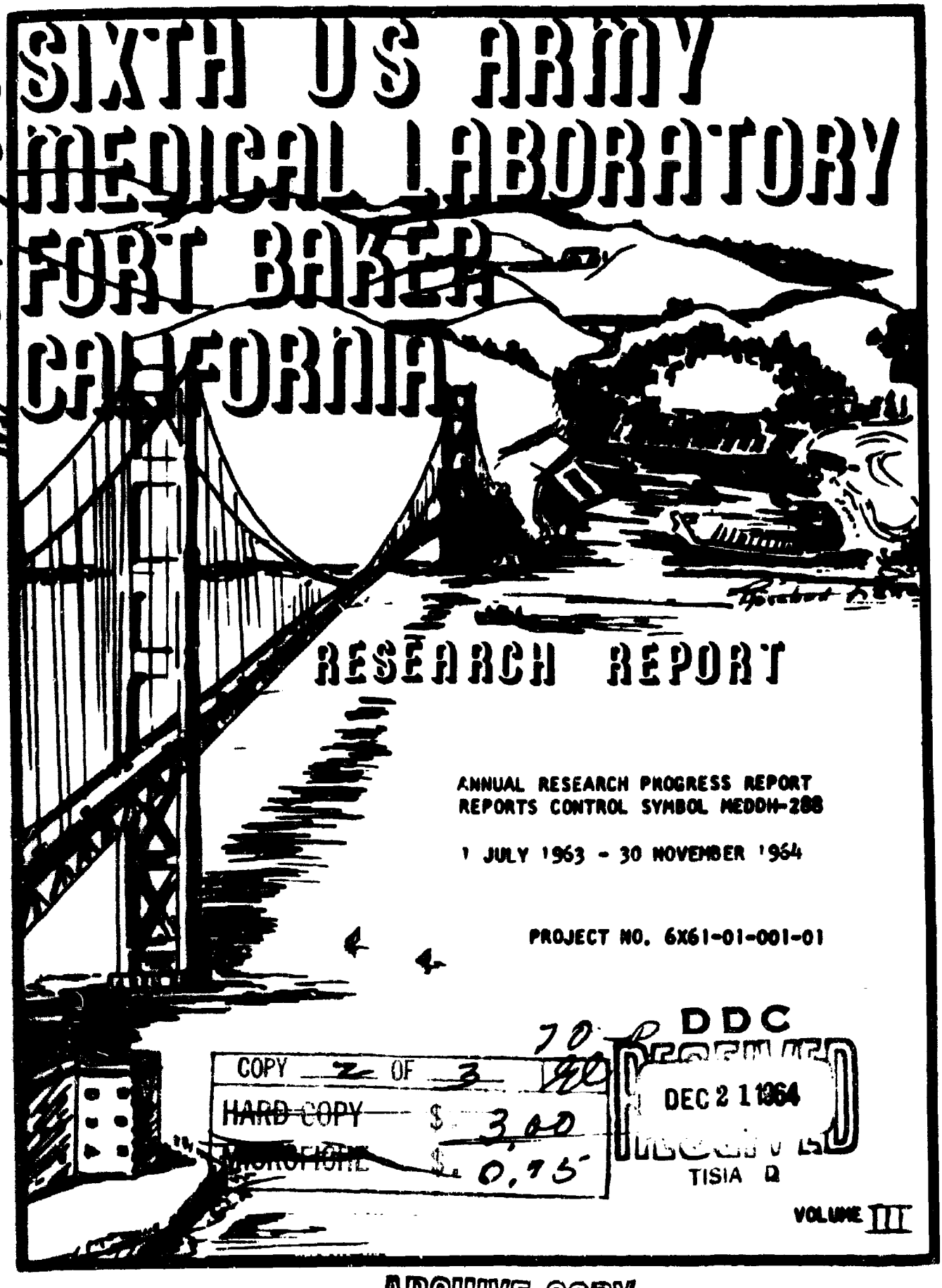

RESEARCH REPORT

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1 JULY 1963 - 30 NOVEMBER 1964

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ANNUAL PROGRESS REPORT

1 July 1963 - - - 30 November 1964

VOLUME III

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ANNUAL PROGRESS REPORT

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FINAL REPORT

Project No. 3A012501A806-11 Accession Number 36,876

Task: Chromosome Analysis and Virus Isolation Studies of Human Embryos and Fetuses

Reporting Installation:	Sixth US Army Medical Laboratory Fort Baker, California Divisions of Pathology and Immunology
Period covered by report:	1 July 1963 through 30 November 1964
Principal Investigators:	Wilkerson, James A., Capt., MC Leibovitz, Albert, Lt Col, MSC
Assistants:	Nakamura, Agnes, B.A. Smith, Raymond P. J., SSG Zoccoli, Steven, SP4
Reports Control Symbol:	MEDDH-288
Security Classification:	UNCLASSIFIED

ABSTRACT

Project No. 3A012501A806-01 — Title: Military Preventive Medicine, Communicable Diseases.

Task No. 1 — Title: Chromosome Analysis and Virus Isolation Studies of Human Embryos and Fetuses

Reporting Installation: Sixth US Army Medical Laboratory, Fort Baker, California; Pathology Division, Virology Section, Immunology Division

Period Covered by Report: 1 July 1963 through 30 November 1964

Authors: James A. Wilkerson, Capt., MC; Albert Laibovitz, Lt Col, MSC; Agnes Nakamura, B.A.; Raymond P. J. Smith, SSG; Steven Zoccoli, SP4

Reports Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

A total of 58 specimens were received, primarily material from abortions in the first trimester of pregnancy, for virus isolation and chromosome studies. No significant virus agents were isolated. Fifty-four of the specimens were processed for establishing cell cultures; 21 failed to grow, 23 regressed before they could be successfully karyotyped, and 10 were karyotyped. Two (2) abnormal karyograms were found: A trisomy of group "E:" (Group 16 to 18) and a subtriploid (modal count of 66).

Peripheral plasma cultures were performed on 30 individuals with karyograms obtained in 25 cases. Abnormal findings were obtained in 7 cases (Down's syndrome: 5; Turner's syndrome: 1, translocation: 1).

This study has enabled the perfecting of techniques to offer chromosome studies as a diagnostic aid to the Armed Forces medical facilities of the Sixth US Army.

BODY OF REPORT

Project No. 3A012501A306-01 Title: Preventive Medicine, Communicable Diseases

Task No. 1 Title: Chromosome Analysis and Virus Isolation Studies of Human Embryos and Fetuses

Description:

Approximately ten percent of all human pregnancies fail to produce a viable term infant. These studies were undertaken in an effort to evaluate the role of chromosomal abnormalities and viral infections in this wastage (Kibrick: Perspectives in Virology, Vol. II, Edited by Morris Pollard, Burgess Pub. Co. Mpls, page 140).

MATERIALS AND METHODS

Collection of specimens:

Liaison was established with the Obstetrical and Gynecological Services of Letterman General Hospital and the Pathology Service of Marin General Hospital for the rapid collection of abortuses. Each installation was supplied with sterile Mason jars containing Puck's Saline "A" plus 1,000 units of penicillin and 1,000 mcg of streptomycin per ml. A questionnaire form to be filled out on each case was to be submitted with each case (see enclosure #1 for questionnaire for formula for Puck's Saline "A"). This installation received telephonic notification of each specimen and immediately dispatched a messenger to obtain the specimens.

Abortuses obtained after 2200 hours were placed in the refrigerator and collected the following day.

Processing of specimens:

All specimens were processes in the Pathology Division. Careful search was made for embryonic tissue which was segregated and placed in Medium L-15 (Leibovitz, A. Amer. J. Hyg. 78: 173, 1963) for tissue growth studies and chromosome analysis. When sufficient material was available, portions of embryonic tissue were also segregated in a separate vial for virus isolation studies. When insufficient known embryonic tissue was discernible, available tissues were utilized for virus isolation studies. Materials for tissue cell growth and virus studies were submitted to the Virology Section for processing.

Techniques for tissue dispersion for tissue cell growth:

a. Non-enzymatic method: When only small fragments of embryonic tissues were available, attempts were made to obtain growth by mechanical dispersion alone. Tissues were finely minced with Bard-Parker #11 knives until the fragments were 1 cubic millimeter or less in size, suspended in medium #15 plus 20 percent fetal calf serum and dispensed in sterile 2 ounce prescription bottles. These bottles were left undisturbed for at least 5 days and then carefully observed for starting cell growth. If none was observed, the bottles were replaced in the incubator and observed again at 5 day intervals. When growth commenced, the supernatant fluid and remaining debris were removed and the cells fed 3 times weekly with medium L-15 plus 20 percent fetal calf serum. When cells grew rapidly, the full bottles were harvested in a 0.05 percent typsin-pancreatin-versene solution (see below) and inoculated into 8 or 16 ounce prescription bottles. On adequate growth in these bottles, the harvest was split in a 2 for 1 distribution, one bottle for chromosome analysis and one bottle to preserve the cell strain for other studies; i.e., latent virus agents and probable use as a diploid strain in the virus diagnostic section.

b. Enzymatic dispersion method: Material minced as above with Bard-Parker #11 knives as above and then transferred to a sterile, screw-cap, 50 ml centrifuge tube containing 10 ml of a 0.25 percent trypsin-pancreatin solution (see below). The enzyme-tissue suspension was placed, at room temperature, on a 180 revolutions per minute shaker for 10 minutes. Placing of the tube in a stationary rack for several minutes permitted the heavy particles to settle leaving a hazy supernatant fluid. This supernate was aspirated for further processing and fresh enzyme solution was placed on the remaining tissue for further shaking at 10 minute intervals. The hazy supernatant fluid was placed in a 15 ml sterile, screw capped test tube, centrifuged at 1,000 rpm for 5 minutes in a horizontal centrifuge yielding a clear supernatant which was discarded and a packed cell suspension which was resuspended in 10 ml of outgrowth media (Medieum L-15, 20 percent fetal calf serum) and dispensed in a 2 ounce screw cap prescription bottle. Each 10 minute yield was so treated until the enzyme action failed to yield a hazy supernatant fluid. Growth of clusters of cells was usually evident within 3 days in one or more of the bottles. Once growth was evident, the supernatant fluid and remaining debris were removed and fresh out-growth media was inoculated. The media was then routinely changed every 3 to 4 days until the cell-sheet completely covered the side of the bottle. The sheet was harvested with a pancreatin-trypsin-versene solution (see below) and inoculated into a 16 ounce bottle. Upon filling this size bottle, the cells were harvested and carried forward on a 2 for 1 split, one bottle being used for chromosome studies and the other to preserve the strain for observation of possible latent virus activity and for use as a "diploid cell strain" for routine virus isolation studies in the

diagnostic section of the division.

(1) Enzyme solutions for cell harvesting.

(a) Pancreatin-Trypsin for original tissues: An 0.25 percent pancreatin-trypsin solution was found to yield quite consistent outgrowth of cells from the usually small pieces of tissue obtained for this study. The formula per liter is as follows:

Trypsin (Difco, 1:250)----------	2.50 gm
Pancreatin (Nutritional Biochemical)----------	2.50 "
NaCl----------	8.00 "
KCl----------	0.40 "
Na_2HPO_4 (anhydrous)----------	1.40 "
Phenol Red (0.4% solution)----------	2.50 ml
Distilled water, qs to----------	1000.00 ml

Mix on magnestir for 30 minutes. Prefilter through Gooch crucible using fine filter paper under vacuum pressure. Millipore filter sterilize. Distribute desired aliquots. Final pH should be about 7.6 Store in deep freeze (at least -20°C).

(b) Pancreatin-trypsin-versene solution was used for harvesting of monolayer of cells from bottles: This enzyme solution has a pH of about 7.6. The growth media is removed from the cell culture and replaced by about 5 ml of the enzyme solution (suitable for any size bottle up to 16 ounces) which is hand rolled over the cell growth for 20 revolutions. This material is then poured off and the step is repeated with 5 ml of fresh enzyme solution. This time all but about ½ ml of solution is poured off and the bottle is placed in the 35°C incubator. The cells usually start to slough off within 5 minutes. Ten ml of outgrowth media is added to the bottle, the cells suspended with a pipette and dispersed in fresh bottles or tubes as desired. The formula per liter is as follows:

Trypsin (Difco, 1:250)----------	0.50 gm
Pancreatin (Nutritional Biochemical)----------	0.50 "
Versene (Nutritional Biochemical)---	0.50 "
NaCl----------	8.00 "
KCl----------	0.40 "
Na_2HPO_4 (anhydrous)----------	0.14 "
Phenol Red (0.4% solution)----------	2.50 ml
Distilled Water----qs----------	1000.00 ml

Prepare as enzyme solution above. Note that only 1/10th the disodium hydrogen phosphate is needed to obtain a pH of 7.6 as the versene is highly alkaline.

CHROMOSOME STUDIES ON TISSUE CELL CULTURES

Cell Cultures:

Must be actively growing cells in optimal condition. Full bottle is harvested and inoculated on a 2 to 1 split. In the late afternoon of the 2d post-inoculation day, add 0.1 ml of stock solution of 60 ug/ml colchemid per 10 ml of medium (yields final concentration of 0.6 ug/ml) per bottle. Replace in incubator overnight.

Procedure for Harvesting Cells for Chromosome Studies:

1. Remove supernatant fluid to 50 ml centrifuge tube.

2. To bottle, add 5 ml of 0.05% pancreatin-trypsin-versene solution. Rotate by hand 20 times to assure complete contact with all cells. Place in 37°C incubator for 5 minutes. Harvest cells and add to cell suspension in the 50 ml centrifuge tube. Centrifuge at 1,000 rpm for 5 minutes. Discard supernatant.

3. Add 5 ml Somer's sucrose solution (3.42% sucrose, .008% $CaCl_2$, 0.002% NaCl) or 0.2% buffered saline solution prewarmed to 37°C (see peripheral blood SOP). Carefully suspend cells with pipette. Place on mechanical shaker for 30 minutes (about 150-180 revolutions per minute).

4. Add 5 ml modified Carnoy's solution (3 parts methyl alcohol plus 1 part glacial acetic acid----this is to be made fresh on the day of use) and carefully mix. Allow to stand at room temperature for at least 10 minutes. Centrifuge at 1,000 rpm for 5 minutes and discard supernatant.

5. Add 5 ml fresh Carnoy's solution and carefully resuspend cells. Allow to stand at least 10 minutes. Centrifuge at 1,000 rpm/5 minutes. Carefully pour off all but ½ ml of the supernatant. Resuspend cells.

6. Place chemically clean slides on flat sheet of dry ice. While slide is becoming lightly frosted, pick up about 2 inches of cell suspension in a capillary pipette. Take slide off ice and allow it to start to thaw. When thawing starts on the bottom and top of frosted area, drop cell suspension on; the fluid should spread rapidly on the slide. Immediately flame which should start the methyl alcohol to burn. The fluid on the slide will contract into small balls. Shake vigorously and the slide will appear completely dry.

7. Stain in 1:21 Giemsa stain.

PERIPHERAL WHITE BLOOD CELL CULTURES FOR CHROMOSOMES

Principle:

Technique based on knowledge that white blood cells will actively proliferate in Medium 15 in the presence of phytohemagglutinin "P." The most active mitosis takes place from 68 to 76 hours after inoculation and rapidly falls off after 86 hours. Therefore work must be planned to take advantage of this narrow range of optimal mitosis. Colchicine, final concentration of 5×10^{-8}M, is placed on the culture on about the 69th hour after inoculation and allowed to stay on for only 5 hours to arrest the cells in metaphase and then the cells are harvested.

Reagents:

Heparin: Usually obtained as the sodium salt containing 110,000 units per gram. Dissolve one gram in 110 ml of medium 15 (without glutamine or serum) to yield 1,000 units/ml. Distribute in 1 ml amounts in 23 ml vials, properly label, and store in refrigerator. Standard item, FSN-6505-153-9740, containing 1,000 u/ml, is also useable.

Phytohemagglutinin "P" (Difco): Received as lyophilized powder that is reconstituted in 5 ml of phosphate buffered saline (Difco's Hemagglutination buffer). Each lot of this chemical must be standardized as its potency will have a profound effect on the mitosis of the cell. Titrations are usually carried out from 0.02 to 0.05 ml per 10 ml of blood (increments of 0.005 ml); most batches are optimal at about 0.025 ml/10 ml blood.

Medium L-15, complete with glutamine and 20% fetal calf serum.

Distribute 10 ml in 2 oz prescription bottle.

Giemsa stain (Coleman and Bell Giemsa for Blood Stain). Add 0.75 gram to 50 ml glycerine, C.P. Place in 60 degree water bath and mix frequently for a 24-hour period. Add 50 ml methyl alcohol. Mix well. Allow to age for several days. Filter. Use 1 part of stock stain to 20 parts of distilled water and stain slides for 30 minutes.

0.2% Buffered Saline: NaCl: 2.0 gm, KCl: 0.4 gm, Na_2HPO_4: .14 gm, H_2O: 1,000 ml. Autoclave 121°C/15 minutes to sterilize.

Carnoy's Solution: Prepare fresh and store in refrigerator. Prepared by adding 1 part of glacial acetic acid to 3 parts of C.P. Methyl Alcohol.

Procedure:

Use 10 ml syringe with 20-gauge needle. Draw up heparin solution

from the 23 ml vial and wet inner barrel of syringe and then expel heparin back into the 23 ml vial. Carefully draw 10 ml of peripheral blood, remove needle, and inject the blood into the heparin containing vial. Mix well and then place vial on sheltered area on table top and allow the red cells to settle. In 1-3 hours sufficient plasma is available for the cultures. Sedimentation can be accentuated by placing in 37°C incubator.

a. Draw off plasma carefully with a DISPOSABLE CAPILLARY PIPETTE (a capillary pipette allows withdrawal of plasma without picking up any red cells) using a rubber bulb for suction. However, slight contamination with red cells will not affect results.

b. Immediately inoculate, in duplicate, 0.75 to 1.50 ml of plasma into Medium L-15 bottles.

c. Incubate at 35-37°C for 69-72 hours. Evidence of growth is noted by granular-like material floating in media. Shake at intervals to help break up these clumps.

d. Harvest cells by transferring culture to a 15 ml centrifuge tube. Spin at 800 rpm/10 minutes. Discard supernatant.

e. Add 5 ml medium L-15 to the culture bottle. Gently swirl to remove any cells adherent to the bottle. Decant into the centrifuge tube containing the sedimented cells. Gently suspend the cells by tapping on the side of the tube. Recentrifuge at 800 rpm/ 5 min.

f. Remove all but 0.5 ml of the supernatant. Resuspend cells again by tapping the bottom end of the tube while holding the top with thumb and forefingers of other hand.

g. Add prewarmed to 37°C 0.2% NaCl BSS solution (4½ cc). Gently resuspend the cells, and place on shaker (180 revolutions/ minute) for 20 minutes.

h. Add 5 ml of chilled Carnoy's solution. Allow to stand for 10 minutes.

i. Centrifuge at 1000 rpm/5 minutes. Aspirate the supernatant. Add 5.0 ml of fresh fixative. Resuspend cells. Let stand 10 minutes. Centrifuge at 1,000 rpm/5 minutes.

j. Remove all but 0.5 ml of supernatant, resuspend cells gently with capillary pipette. If cells are in smooth suspension, they are ready for slide making. If clumps are still prominent, repeat adding of 5 ml of fixative, 5 minutes in refrigerator and recentrifuging. If clumps persist, allow cells to stand for 10 minutes and carefully remove supernatant for slides. Amount of fixative to be used in final preparation before slide making depends on concentration

of cells--should be diluted to yield a hazy suspension (usually 0.4 to 0.3 ml).

k. Slides: Method of Fox and Zeiss: Precleaned slides placed on block of dry ice (flat piece) until frosted. Remove from dry ice and allow to start to thaw. As thawing is evident around edges of slide (including bottom), hold slide at about 45 degree angle and several drops of the cell suspension are placed thereon (these should spread rapidly). Gently flame and the excess methyl alcohol will burn off and the liquid will suddenly retract into a ball. Rapidly shake in the air and the slide will become dry. Allow to dry for several minutes additional before staining.

l. Stain in 1:21 Giemsa for at least 10 minutes. Rinse in distilled water. Air Dry. Examine under low power. If slides are satisfactory, mount in Harlesco or Permount.

m. Satisfactory spreads are microphotographed. At least 10 photos per patient for modal count and karyotyping.

Technique of virus isolation studies:

Tissues were ground with a mortar and pestle and prepared as a 10 percent suspension with 10 percent skim milk. This suspension was centrifuged at 4,500 rpm in a refrigerated centrifuge (international, PR-1) and the supernate used for virus isolation studies.

The supernate was further diluted 1 in 4 in Medium 15, 2 percent fetal calf serum containing 100 u penicillin, 100 mcg streptomycin, and 3 mcg fungazone per ml. Sufficient material was prepared to inoculate 2 ml per tube of pairs of different cell cultures in use at the time. Usually 5 different cell strains or lines were tested with each specimen which included human malignant lines (HEp-II and/or Hela), human diploid cell strains (lung, kidney) and one primate line (rhesus or african green monkey kidney). At times during this study, other animal strains were tested as rabbit kidney and embryonic rhesus lung.

At the start of this study, the medium was changed on each cell culture 3 times per week and each culture was followed from 15 to 30 days before it was blind-passed. Blind passage was only performed when the cells started to degenerate. At least one blind passage was performed with each cell culture. As progress was made in development of a maintenance medium that permitted long term growth of the cells without feeding (see research report "Culture media for the growth and maintenance of tissue cells" in this volume), malignant lines could be followed without feeding from 7 to 12 days and diploid lines from 21 to 30 days (or longer) before blind passage was necessary.

Primary outgrowths of the various tissues under study were passaged as many times as possible before they regressed and they

were observed for possible outcropping of latent agents. When cell cultures regressed, they were harvested and inoculated into the routine cell cultures for possible virus isolation.

RESULTS

Outgrowth of tissues for chromosome determinations:

Relationship of time of enzyme treatment to age of embryo on outgrowth of tissue cells. It was observed early in this study that there was a direct relationship between the age of the embryo and the length of time for enzymatic treatment to obtain growth of cells. Invariably, embryos obtained in the first trimester of pregnancy showed outgrowth of cells in the 10, 20 and sometimes the 30 minute pancreatin-trypsin harvests and rarely thereafter, whereas the tissues obtained from stillbirths rarely showed growth until at least the 30 minute harvest and usually the best yields were obtained in 60 minute to the 120 minute harvests. The fragments that were not enzymatically treated, but only mechanically dispersed, yielded outgrowths haphazardly, and this may be related to whether fetal or probably maternal tissue was being dispersed.

Relationship of condition of embryo to cell outgrowth. In many instances the embryos obtained in the first trimester of pregnancy were either blighted or in a state of decay. Although initial outgrowth was obtained in 7 of 8 "blighted" embryos, all of these regressed before they could be successfully karyotyped. Only 1 of the 3 "macerated" embryos showed initial outgrowth and was successfully karyogrammed as normal. Initial outgrowth was obtained in 6 of 9 embryos that appeared grossly normal, but only 2 were successfully karyotyped and both had an apparent normal karyogram. See table 1.

No fetus could be detected in 24 specimens obtained in the first trimester of pregnancy. In 15 cases, fetal membranes were discernible and processed with 9 showing successful initial outgrowths; 5 of these regressed before being karyotyped and 4 were successfully completed. Of these 3 were apparently normal, but one piece of amnion yielded a "stable" cell strain with a subtriploid chromosomal count of 66. This culture was passed 68 times before it was frozen down and it retained the same pattern. Grossly the cell appeared as a normal amnion cell. Of interest, the mother was again pregnant when she was located at a different station and this time she yielded a full term infant that died almost immediately after birth. Specimens for chromosome studies have just been received and are under study. See table 1.

No definite fetal tissue could be discerned in 9 cases and probable decidua was cultured with 6 yielding a successful initial outgrowth but all regressing before they could be karyogrammed. See table 1.

Lung and kidney tissue were obtained from 5 stillbirths; 4 of these showed initial outgrowth and 3 were successfully karyotyped. Two were apparently normal but the third yielded a modal count of 47 chromosomes (90 percent of the cells) due to a trisomy in group "E" (16 to 18 in the Denver system classification). See table 1.

Most of the cultures that regressed showed initial outgrowths of small whorls of epithelial-like cells that appeared to progress to small colonies of about 20 cells and then to cease growing and to degenerate. When fibroblast-like cells were obtained, growth usually continued until the bottle was filled, but on subculture a granular-type cell was noted that failed to grow normally. All attempts to obtain cells in mitosis failed using short term colchicine treated cells (5 hours) to long term colcemid treated cells (18 to 24 hours).

All cultures that were successfully karyotyped were fibroblast-like cells with the exception of the amnion-like cell that yielded the subtriploid chromosomal count noted above.

When the "word" got out that this station was doing research in chromosome studies, physicians began requesting aid in the study of viable patients with probable congenital defects. To date 30 such studies have been made using the peripheral blood plasma technique (see methods) on 17 patients with probable congenital defects (infants to teenagers), 3 with leukemia, and 10 parents for possible parental transmission of abnormal chromosomal factors. See table 2.

Mitosis with successful karyograms was obtained in 26 of the above cases; 7 of the patients with probable congenital defects yielded an abnormal karyogram: Turner's syndrome: 1; Down's syndrome: 5; a teenager with multiple defects and some mental retardation showed a translocation in Group "D" in about 5 percent of his cells (see figure #1). One of the infants with Down's syndrome had a pseudo-normal count of 46 chromosomes but on karyogram demonstrated that the extra group 21 chromosome had translocated to the group 15 chromosome (see figure #2).

It is of interest that 4 of the 5 mothers having infants with Down's syndrome are in their early twenties and this is their first or second child. The fathers are also in their twenties. The fifth mother is in her late thirties and had had two previous miscarriages; this is her first viable child.

Viral Studies:

Although several virus agents were isolated from tissues obtained from abortions in the first trimester of pregnancy, no significant way could be determined to establish that they were the actual etiological agent of the abortion. Difficulty was had in obtaining acute and convalescent sera on the mothers although

numerous requests were made for same. Coxsackie group B, type 1, was isolated in 2 instances; Poliovirus type 1 in 4 instances (however, Sabin vaccine was being administered at this time and there is no way of ruling out fecal contamination of the specimens obtained), and Herpes simplex was obtained in 1 instance. It is of interest that only in 1 of these cases was initial outgrowth of cell cultures successful (a poliovirus was isolated from the tissue fragments remaining for virus isolation studies) and there was no evidence of poliovirus being present in the cell outgrowth. The karyogram was normal.

Cell cultures that regressed did not show evidence of cytopathogenic changes usually associated with virus infection. No known virus agent could be demonstrated by cytopathogenic effect or by the hemadsorption technique in the original cultures or in subcultures into other cell lines or strains.

DISCUSSION

This short term study (the senior author has left the Service) unfolds the difficulty in the study of material obtained from the first trimester of pregnancy for chromosomal analysis. In most instances, the fetus has been dead for varying periods of time prior to being aborted and is usually blighted or decaying and attempts to obtain outgrowths of such material are usually unsuccessful. When initial whorls of cells are obtained, they usually regress. Improvements in cell husbandry techniques may help to overcome this problem. In many instances the fetus is lost and the value of culturing the tissues obtained is questionable although one such study did yield a sub-triploid cell from a piece of amnion. The basic thesis of this research project has not been answered although there is some evidence that obvious chromosomal abnormalities and possible virus infection may both play a role in this wastage.

TABLE 1

Results of attempting to culture tissues from abortions and stillbirths

TYPE OF SPECIMAN CULTURES	NUMBER OF SPECIMENS	POSITIVE INITIAL GROWTH	REGRESSED	KARYOTYPED	ABNORMAL KARYOTYPE
1. Fetus					
a. "Blighted"	8	7	7	0	
b. "Normal"*	9	5	4	2	0
c. Macerated	9	1	0	1	0
2. No fetus					
a. Fetal membranes	15	9	5	4	1 (56)**
b. Decidua	9	5	6	0	
3. Stillbirth	5	4	1	3	1 Trisomy**
TOTALS	54	33	23	10	2

* "Normal" fetus: No gross evidence of decay.

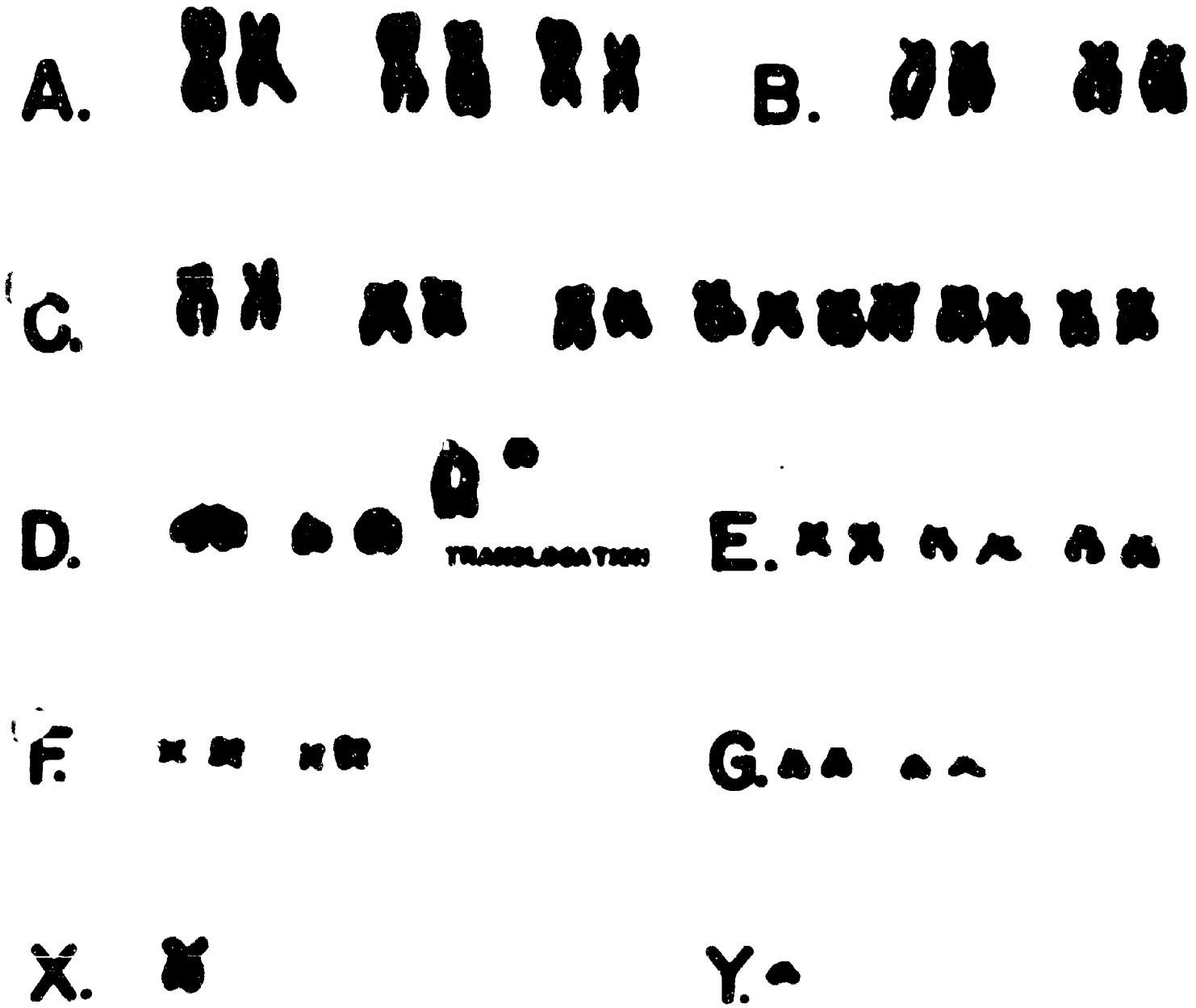
**Abnormal karyotypes: a. Fetal membrane (amnion): Yielded "stable" cell cultures with 66 chromosomes. b. Stillbirth: Trisomy in group "E" (16 to 18).

TABLE 2

Results of peripheral blood chromosome studies

SOURCE OF SPECIMENS	NUMBER	MITOSIS POSITIVE	NORMAL KARYOTYPE	ABNORMAL KARYOTYPE*
1. Patient (suspected congenital defect)	17	15	8	7
2. Leukemia	3	1	1	0
3. Parents	10	10	10	0
TOTALS	30	26	19	7

* ABNORMAL KARYOTYPES: Turner's syndrome: 1; Down's syndrome: 5, Translocation 1.



FIGURE

Maria Martinez CH-181

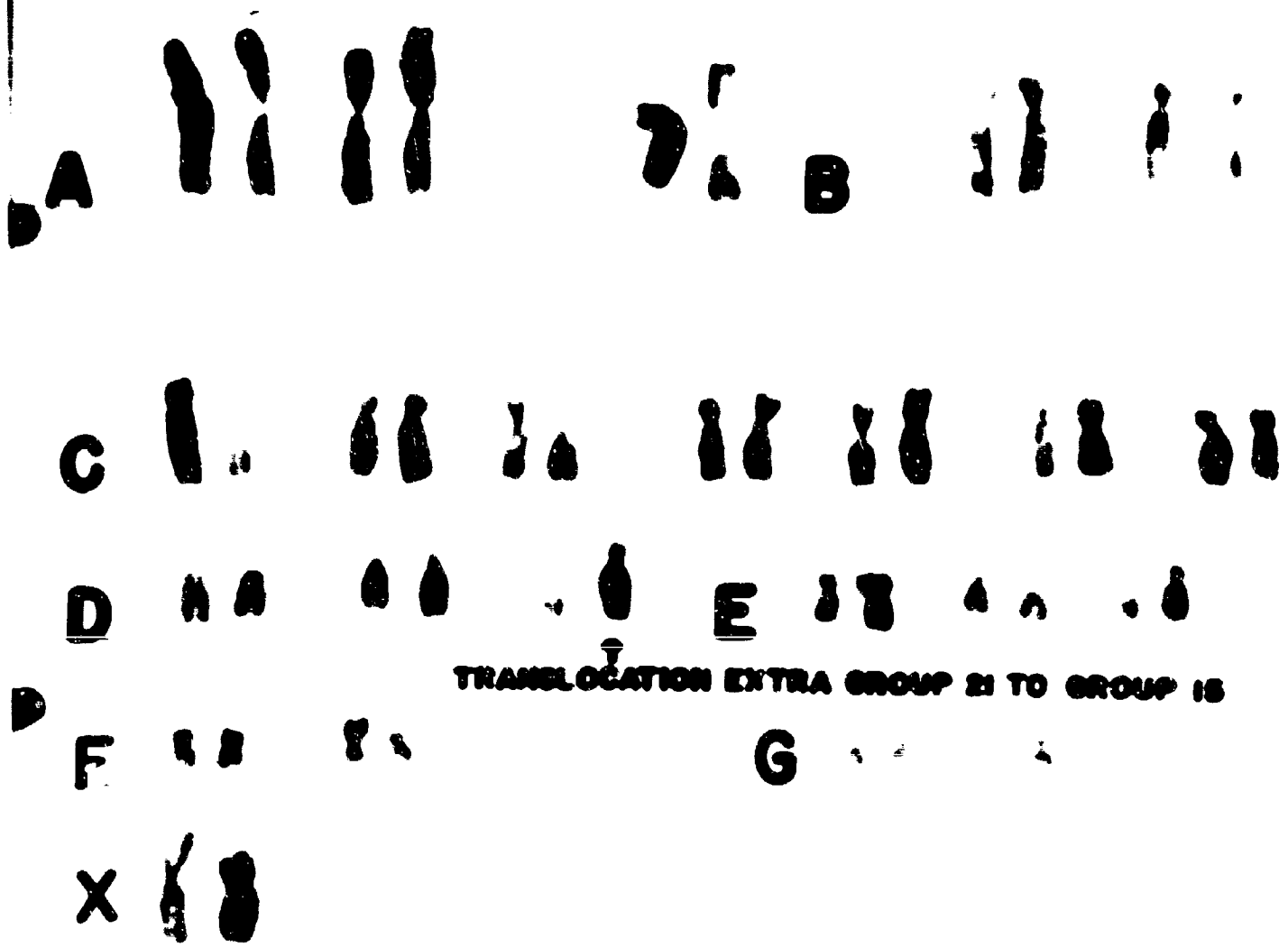


FIGURE 2

FORM FOR ABORTUSES FOR

VIRAL AND CHROMOSOME STUDIES

Hospital: _____ Ward: _____

Name: _____ Age: ____ Race _____

Hospital Number: _____ Attending Physician: _____

Date of Abortion (or Curettage): _____ Time: _____

Gestational History: Gr. _____ P. _____ Ab. _____

Present Pregnancy: LMP _____

1. Complications:

2. Abortion History (Cramps, Chills and Fever, Bleeding, Duration, etc.):

3. History of Viral Illness of ANY TYPE during Pregnancy.
   Yes. No.

   Date:

   Nature of Illness: (Include Diagnostic Studies):

4. Known Contact with Other Individuals with ANY TYPE of Viral Illness, including Immediate Family.

   Date:

   Nature of Illness:

5. Vaccinations:
   During Present Pregnancy:
   Agent:

   Date:

   Prior to Present Pregnancy:
   Agent (s):

   Date(s):

Other Significant Medical History:

Note: Please fill in all spaces. Use "None" or "Not Applicable" (NA) as necessary. If more space is required please use reverse side of this sheet.

6AML Form #85 (30 Dec 63)

METHOD OF COLLECTION OF SPECIMENS

1. Virus studies only:

a. Products of conception (abortuses or curettings) are collected as aseptically as possible, placed in sterile, wide mouth, screw cap jars, sealed tight with tape and/or paraffin, and frozen under dry ice. Material is maintained in a frozen state until it arrives at the 6th USAML.

b. Acute and convalescent sera: To relate any virus isolated to possible infection of the mother as the cause of the interrupted pregnancy, both acute and convalescent serum specimens from the mother are necessary. The acute specimen is obtained immediately, the convalescent about 21 days later. At least 10 ml. of clotted, whole blood for each specimen is required.

2. Specimens for both virus studies and chromosomes studies:

a. ABORTIONS: Collect all products of conception available.

b. FETUSES AND STILLBORNS: If the specimen obtained is small enough to be considered a "surgical" specimen, the entire fetus is desired; if large enough to require autopsy, LUNGS and KIDNEY should be submitted.

c. CARRIER MEDIUM AND BOTTLES: Sterile, wide mouth, screw cap bottles and Puck's Saline "A" are used to receive the specimens. This material can be furnished by the 6th USAML on request or produced locally by laboratories.

Formula for Puck's Saline "A":

	PER LITER
Sodium chloride	8.00 gm.
Potassium chloride	0.40 gm.
Sodium bicarbonate	0.35 gm.
Glucose	1.00 gm.
Distilled water	1000.00 ml.
Phenol red (0.4% solution)	2.50 ml.

(1) Dissolve all ingredients. Dispense in 100 ml. aliquots in screw capped bottles. Autoclave at 121°C (15 pounds pressure) for 10 minutes. Add to each bottle 1 ml. of a solution containing 10,000 mcg. of penicillin and streptomycin per milliliter.

d. STORAGE AND SHIPMENT OF SPECIMENS: All specimens are to be placed immediately in the refrigerator and plans made to transmit them to the 6th USAML within 24 hours after receipt. The specimens should be placed in a water-tight container and packed with WET ICE in sufficient quantities for the specimen to arrive at the 6th USAML

under refrigeration. Stations distant from this laboratory can transmit by airmail special delivery by timing their packing of the specimens to coincide as close as possible to the mailing schedule. Courier service should be used whenever feasible.

e. PRECAUTIONS IN COLLECTION OF SPECIMENS: To help maintain tissue in a viable state, care must be taken to avoid contact with toxic substances such as disinfectants, soaps, formaldehyde, etc. Sterile precautions should be maintained as far as is reasonable.

f. ACUTE AND CONVALESCENT SERA: Essential as noted above for virus studies.

ANNUAL PROGRESS REPORT

Project 6X61-01-001-01. Preventive Medicine - Communicable Diseases

Task 1, Studies on Penicillin Treatment Failures of Gonorrhea

Reporting Installations:	Sixth US Army Medical Laboratory Fort Baker, California
	US Army Hospital Fort Huachuca, Arizona
Period Covered by Report:	1 July 1963 through 30 June 1964
Principal Investigators:	Adrian D. Mandel, Lt Col, MSC Edward Kopplin, Jr., Capt, MSC* Robert S. Croissant, Capt, MC* George J. Omi, B.S.
Reports Control Symbol:	MEDDH-288
Security Classification:	UNCLASSIFIED

* US Army Hospital, Fort Huachuca, Arizona

ABSTRACT

Project 6X61-01-001-01 — Title: Preventive Medicine - Communicable Diseases

Task No. 1 — Title: Studies on Penicillin Treatment Failures of Gonorrhea

Reporting Installations: Sixth US Army Medical Laboratory, Fort Baker, California; US Army Hospital, Fort Huachuca, Arizona

Period Covered by Report: 1 July 1963 through 30 November 1964

Authors: Adrian D. Mandel, Lt Col, MSC; Edward Kopplin, Jr., Capt, MSC; Robert S. Croissant, Capt, MC; George J. Omi, B.S.

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

In an effort to determine the role of Mimae-Herellea organisms in penicillin-resistant gonorrhea, a new medium for the selective isolation of these organisms was developed. In a series of 80 cases of gonorrhea, including 4 cases that were not successfully treated with penicillin, Mimae-Herellea organisms were isolated only once, and in that instance were not associated with a treatment failure.

BODY OF REPORT

Project 6X61-01-001-01 Title: Preventive Medicine - Communicable Diseases

Task No. 1 Title: Studies on Penicillin Treatment Failures of Gonorrhea

Description:

During the past year, this study was handicapped by a shortage of personnel needed to isolate and perform penicillin-sensitivity testing of strains of N. gonorrhoeae from cases of gonorrhea, and this portion of the study was not done. Emphasis was placed on the refinement of a selective medium for the isolation of Mimae-Herellea organisms, reportedly associated with penicillin-resistant gonorrhea.

Progress:

Studies on Penicillin-Resistant Gonorrhea:

a. An absence of trained personnel resulted in the discontinuation of that portion of the project dealing with isolation and penicillin-sensitivity testing of N. gonorrhoeae isolated from cases of gonorrhea.

b. Due to the reported association of Mimae-Herellea organisms with penicillin treatment failures of gonorrhea, an attempt was made to develop a medium that would selectively permit the isolation of these organisms. The study of the role of these organisms has been hindered due to the uncertainty of recognizing Mimae-Herellea organisms in the presence of large numbers of gram-positive cocci and gram-negative rods (usually members of the family Enterobacteriaceae), frequently encountered in urethral and vaginal discharges. After a number of trials, the formulation of the most satisfactory medium was as follows (in grams per liter of distilled water): pancreatic digest of casein (Difco): 15; soy peptone: (Difco): 5; sodium chloride: 5; lactose: 10; maltose: 10; bile salts: 1.25; bromocresol purple: 0.02; and agar: 16. After 24 hrs at 37 C, colonies of Mima and Herellea organisms were pale lavender. All acid producing colonies were yellow, surrounded by a yellow zone. The growth of gram-positive organisms was inhibited. Species of Pseudomonas and Proteus were not inhibited, nor did they produce acid. However, Pseudomonas colonies were easily recognized by their gray-green color and diffusible pigment, when present. If desired, Proteus colonies can be differentiated by the addition of 5.0 g. of phenylalanine and 0.5 g. of ferric ammonium citrate to the medium, in which case Proteus colonies are brown, surrounded by a light-brown zone. A total of 80 patients with gonorrhea was examined for the presence of Mimae-Herellea organisms, using this new

medium. Only one isolation of a strain of Mima was made, and this was not from a patient who was a penicillin treatment failure. This study continues to fail to find support for the supposition that Mimae-Herellea organisms are associated with penicillin treatment failures of gonorrhea.

c. This study is now considered to be terminated. All of the funds have been expended, and a final report will be submitted.

Publications

Mandel, A.D., Wright, K., and McKinnon, J.M., Selective medium for isolation of Mimae-Herellea organisms, J. Bact. 1964, 88: 1524-1525.

ANNUAL PROGRESS REPORT

Project No. 6X61-01-001-01, Communicable Diseases

Task No. 2, Culture Media for Growth and Maintenance of Tissue Cell Cultures

Reporting Installation:	Sixth US Army Medical Laboratory Fort Baker, California Division of Immunology
Period Covered by Report:	1 July 1963 through 30 November 1964
Principal Investigator:	Albert Leibovitz, Lt Col, MSC
Assistants:	Raymond P. J. Smith, SSG Agnes Nakamura, B.A. Hank Blank, B.S. Stephen Zoccoli, SP4 Ronald Shiromoto, B.A.
Reports Control Symbol:	MEDDH-288
Security Classification:	UNCLASSIFIED

ABSTRACT

Project 6X61-01-001-01 Title: Communicable Diseases

Task No. 2 Title: Culture Media for Growth and Maintenance of Tissue Cell Cultures

Reporting Installation: Sixth US Army Medical Laboratory
Fort Baker, California

Period Covered by Report: 1 July 1963 through 30 November 1964

Authors: Albert Leibovitz, Lt Col, MSC
Raymond P. J. Smith, SSG
Agnes Nakamura, B.A.
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Stephen Zoccoli, SP4
Ronald Shiromoto, B.A.

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

Extended studies were made during the past year on the development of a maintenance medium that would eliminate the necessity of feeding cell cultures following inoculation with specimens for virus isolation studies and yet permit long term studies. To enhance isolation of such virus agents that may be inhibited by normal body fluids, as serum, an adequate replacement was found in the use of lactalbumin hydrolysate, polyvinylpyrollidone and fatty acid poor bovine albumin. These studies have largely eliminated the routine labor involved in tissue cell husbandry and has made it possible for small virus diagnostic laboratories to conduct large scale isolation surveys.

BODY OF REPORT

Project No. 6X61-01-001-01 Title: Communicable Diseases

Task No. 2 Title: Culture Media for Growth and Maintenance of Tissue Cell Cultures

Description:

The purpose of the present investigations is to develop media, preferably synthetic, that will enable the rapid growth of both "normal" and "malignant" tissue cell cultures and to enhance the growth of virus agents therein.

Progress:

The development of Medium L-15, which permits the growth and maintenance of tissue cell cultures in free gas exchange with the atmosphere has been described in previous annual reports (Vol. 1, 1962 and Vol. 2, 1963) and has been published (Leibovitz, A., Amer. Jour. Hyg. 78: 173-180, Sep. 1963). This medium has received international recognition and is being presently manufactured in the United States by at least 4 biological houses (Microbiological Associates, Grand Island Biological Company, Difco Laboratories, and Hyland Laboratories). The elimination of bicarbonate as a buffer system and the increase in available amino acids has enabled long term studies of both normal and malignant cell cultures before degeneration sets in. The ability to grow cells in free gas exchange with the atmosphere has eliminated the necessity of capping tubes and bottles prior to inoculation with infectious agents and has thus markedly reduced the manual labor involved in tissue cell husbandry.

During the past year, the serious bottleneck of having to feed tissue cell cultures following inoculation with specimens that may contain virus agents has been eliminated. The necessity of feeding tubes to maintain tissue cell integrity had two serious drawbacks; (1) the manual labor involved and (2) the danger of cross-contamination (most tissue culture laboratories have found themselves in the predicament of suddenly finding the same virus agent in a variety of specimens, regardless of source). Another serious bottleneck, the use of body fluids, especially normal human or animal sera, to help maintain tissue cell integrity over a long period of time, has also been eliminated in tissue cell maintenance. The most obvious interference of sera with virus growth is seen in the isolation of the Myxovirus agents; the mucopolysaccharides and mucoproteins interfere with the demonstration of hemadsorption of red blood cells by the virus agents and thus mask their presence. Actual reduction in virus growth has been noted by many investigators.

MATERIAL AND METHODS

Formula for Maintenance Medium for all Cell Cultures: The following ingredients are added per liter of medium L-15, complete with glutamine and antibiotics but without serum:

5% Lactalbumin hydrolysate*	50.00 ml
10% Polyvinylpyrrolidone**	30.00 ml
1% Bovine albumin, fatty acid poor***	50.00 ml

* Nutritional Biochemical
** Mann Laboratories
***Pentex Incorporated

Preparation of Ingredients:

Lactalbumin hydrolysate:	5% solution per liter
Triple distilled water	970.00 ml
NaCl	8.00 gm
KCl	0.40 gm
K_2HPO_4 (anhydrous)	1.74 gm
Phenol red (0.4% solution)	2.50 ml
Lactalbumin hydrolysate (solubilized)	50.00 gm

Place 970 ml water in Erlenmeyer flask containing a magnetic bar and place on magnestir. Add each ingredient separately and allow to dissolve before adding next ingredient. Allow stirring to continue until a sparkling clear solution is obtained. Sterilize through a millipore filter. Distribute in desired aliquots. Color of solution should be amber with about a 6.7 pH.

Polyvinylpyrollidone (PVP 40)	10% per liter
Triple distilled water	950.00 ml
NaCl	3.00 gm
KCl	0.40 gm
K_2HPO_4 (anhydrous)	1.74 gm
PVP-40 (pharmaceutical grade)	100.00 gm
Phenol red (0.4% solution)	2.50 ml

1. Put in solution and sterilize as for lactalbumin solution above.

2. Final pH is about 6.7

3. Store in refrigerator or deep freeze.

Bovine Albumin, Fatty Acid Poor	4% per liter
Triple distilled water	980.00 ml
NaCl	8.00 gm
KCl	0.40 gm
K_2HPO_4 (anhydrous)	1.74 gm
Bovine albumin, fatty acid poor	40.00 gm
Phenol red (0.4% solution)	2.50 ml

1. Put in solution and sterilize as for lactalbumin solution. Use of pre-filter with millipore filter will facilitate sterilization.

2. Final pH is about 6.6

3. Store in refrigerator or deep freeze.

DISCUSSION

The use of the above medium for maintenance has enabled the relatively long term study of both malignant and normal cell cultures. Uninoculated malignant cell cultures in bottles have been held up to 45 days without feeding and have retained about 50 percent of their viable population which can be readily induced back into the log phase of growth by the addition of regular outgrowth media (Media L-15 plus 10 percent fetal calf serum). Diploid cell cultures, when obtained from active, healthy cells, do not show an untoward degeneration in this period of time.

Cells inoculated with a variety of specimens (throat wash, stool, tissues, spinal fluid, etc.) and placed on roller drums, as to be expected, have a shorter life span. However, malignant cells will survive from 7 days to 21 days (especially Hela cells) and diploid cells from 21 to 30 or more days unless virus activity is evident. This survival is fully as good as cells maintained on serum containing medium without additional feeding.

Cells can be maintained on Medium 15 without serum and without the above supplement, but malignant cells usually start to show degeneration within 7 days and diploid cells start to show retraction within 15 days. The value of the above medium has been repeatedly demonstrated through the isolation of myxovirus agents (especially the parainfluenza agents) and the slow growing picorneviruses.

The elimination of feeding inoculated tubes has enabled this laboratory to quadruple the number of specimens that can be readily handled by the same number of personnel and a large scale survey study was undertaken in addition to the normal work load of the section. In addition, the number of different cell cultures that could be used for investigation of both routine and special studies has been increased from 3 to 5 and thus has enhanced the possibility of successful virus isolations. At the present time, the following cell cultures are in use:

Embryonic human lung, embryonic human skin and muscle, Hela, HEp-II, and Rhesus monkey kidney. All of these cell cultures are being carried serially in medium L-15 plus fetal calf serum; even the Rhesus cell can be carried up to 10 passages before obvious latent agents negate its value for diagnostic purposes.

Interesting isolations during the year was the uncovering of Coxsackie A-16 as the cause of "hand, foot and mouth" disease in children (results to be published as co-author with Captain Richardson, MC, of an Air Force Medical facility) and the isolation of Parainfluenza type I from 3 cases of sudden death (one from the brain and two from the heart's blood obtained at autopsy). This agent was also isolated from a case of pericarditis and from a necrotising lesion of the cornea. As some of these isolates are being made in adults, some revision may have to be made in evaluating the role of this organism in adult disease.

ANNUAL PROGRESS REPORT

Project 3A012501A818-01, Communicable Diseases and Immunology-Communicable Diseases

Task 1, Inhibition of Hexose and Glycerol Utilization by 2-Deoxy-D-Glucose in *Trypanosoma gambiense* and *Trypanosoma rhodesiense*.

Reporting Installation:	Sixth US Army Medical Laboratory Fort Baker, California Parasitology Division
Period Covered by Report:	1 July 1963 through 30 June 1964
Principal Investigators:	John R. Seed, 1st Lt, MSC Mauro A. Baquero, Sp-5 Joseph F. Duda, Sp-4
Reports Control Symbol:	MEDDH-288
Security Classification:	UNCLASSIFIED

ABSTRACT

Project 3A012501A018-01 — Title: Communicable Diseases and Immunology-Communicable Diseases

Task No. 1 — Title: Inhibition of Hexose and Glycerol Utilization by 2-Deoxy-D-Glucose in *Trypanosoma gambiense* and *Trypanosoma rhodesiense*.

Reporting Installation: Sixth US Army Medical Laboratory, Fort Baker, California

Period Covered by Report: 1 July 1963 through 30 June 1964

Authors: John R. Seed, 1st Lt., MSC
Mauro A. Baquero, Sp-5
Joseph F. Duda, Sp-4

Report Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

2-deoxy-d-glucose (D-2-D) was shown to inhibit the fermentation of glucose, mannose, fructose, and glycerol by *Trypanosoma gambiense*. Evidence is presented to support the conclusion that D-2-D inhibits metabolism at least two sites within the Embden-Meyerhof-Parnas pathway. The inhibition of both hexose and glycerol utilization by D-2-D was reversed by the addition of glucose; however, inhibition of glycerol utilization was much more sensitive to reversal. A second analogue of glucose, glucosamine, was also capable of inhibiting glycerol utilization but had no apparent effect on the fermentation of glucose. The structural requirements of compounds capable of acting as inhibitors of hexose and glycerol utilization as well as those compounds capable of relieving glycerol inhibition were shown to be highly specific.

At present, the exact enzymatic sites inhibited by either D-2-D or glucosamine is unknown. However, the possibility that D-2-D inhibits the transport of substrates across the cell membrane is discussed.

This paper has been submitted to EXPERIMENTAL PARASITOLOGY for possible publication.

BODY OF REPORT

Project No. 3A012501A818-01 Title: Communicable Diseases and Immunology-Communicable Diseases

Task No. 1 Title: Inhibition of Hexose and Glycerol Utilization by 2-Deoxy-D-Glucose in *Trypanosoma gambiense* and *Trypanosoma rhodesiense*

Description:

The African trypanosomes utilize three different carbohydrates: glucose, mannose and fructose and one alcohol, glycerol (von Brand, T., 1952). These carbohydrates (and glycerol) are solely utilized via the Embden-Meyerhof-Parnas (EMP) pathway (Grant, P.T., and Fulton, J.D., 1957). Although these substrates share a common pathway, they are utilized at very different rates. In order to explain the differences in rate of hexoses utilization, it was assumed that the rate limiting step must be one of the initial steps in the EMP pathway, i.e., transport of hexoses across the cell membrane or phosphorylation of hexoses by hexokinase. Since it is believed that the glucose analogue, 2-deoxy-d-glucose (D-2-D), inhibits a hexose transport system in yeast (Scharff, T.G., 1961, and Scharff, T.G., and Kremer, III, E.H., 1962), the effect of D-2-D on the fermentation of various substrates by trypanosomes was examined.

This paper describes the effect of two glucose analogues (D-2-D and glucosamine) on the metabolism of glucose, mannose, fructose, and glycerol in *Trypanosoma gambiense* and *Trypanosoma rhodesiense*.

Progress:

MATERIALS AND METHODS

Strains of trypanosomes.

The (XaR) strain of *Trypanosoma rhodesiense* has been described previously (Seed, J.R., 1963). The Wellcome TS strain was isolated from a patient in 1921 and has subsequently been maintained in rodents. These strains were syringe passaged every second day through 20 gram female mice.

Isolation of trypanosomes.

Mice were bled by heart puncture at the height of infection, using 1% sodium citrate (W/V) as the anticoagulant. The suspension was centrifuged at 1300 RPMs in an International Clinical Centrifuge for 4 minutes. After centrifugation, the serum and the trypanosome

layer was removed from the underlying red cell layer with a Pasteur pipette. This suspension was then centrifuged at 1900 RPMs for 4 minutes in the clinical centrifuge and the serum layer removed. The trypanosomes were resuspended in Ringer's phosphate (0.1M NaCl, 0.06M KCl, 0.02M $MgSO_4$, and 0.025M phosphate buffer, pH 7.2) containing either a hexose (0.01M) or glycerol (0.01M) and centrifuged again for 4 minutes at 2780 RPMs in the clinical centrifuge. The trypanosome layer was removed and resuspended at a concentration of 2.5 to 3.5 x 10^7 cells/ml (0.3 to 0.4 mg Protein/ml) in Ringer's phosphate plus substrate. The suspension of trypanosomes was almost completely free of any contaminating erthrocytes, white blood cells or platelets.

Oxygen uptake.

Oxygen uptake was measured in a conventional Warburg apparatus in air at 31°C. Each flask contained 2.8 ml of fluid. This included 0.2 ml of a 20% KOH (W/V) solution in the center well; 0.1 ml of MEM amino acid mixture; 0.1 ml of a MEM vitamin mixture, and 1.8 ml of the trypanosome suspension in the main compartment. The manometer flasks were shaken at 120 oscillations/minute.

Biochemical assays.

Hexokinase was assayed according to methods described previously (Seed, J.R., 1963). Trypanosome extracts were obtained by suspending 0.2 ml of packed trypanosomes in 1.0 ml distilled water with the aid of a teflon homogenizer. The suspension was centrifuged at 3600 RPMs for 15 minutes in a clinical centrifuge. The supernatant was used as the enzyme extract. Protein of the trypanosome suspension was assayed by the method of Lowry et al. (1951).

Materials.

2-deoxy-d-glucose (M.A.), 2-deoxy-d-ribose (M.A.), d-glucosamine hydrochloride (M.A.), L(-)sorbose (M.A.), were all obtained from Mann Research Laboratories, New York 6, New York. D(+) galactose (Sigma grade) was obtained from the Sigma Chemical Company, St. Louis 18, Mo. All hexoses listed are of the highest quality available commercially. MEM essential amino acid solution and MEM vitamin solution were obtained from Microbiological Associates, Bethesda, Maryland.

RESULTS

It was found that 2-deoxy-d-glucose (D-2-D) inhibited the oxygen consumption of T. gambiense in the presence of glucose, mannose, fructose and glycerol (Figure 1). This was not due to an inhibition of the hydrogen transport system, since D-2-D not only inhibited oxygen uptake but also acid accumulation. In addition, D-2-D had no effect on oxygen consumption in cell-lysates. Similar results have also been obtained with T. rhodesiense, except that D-2-D had no effect on the fermentation of glucose (Figure 2) and glucose, itself, inhibited the utilization of glycerol.

It was found that the addition of sufficient glucose would overcome the inhibition of glucose or glycerol fermentation by D-2-D (Figure 3 and Table 1). However, Table 1 also showed that it required at least five times more glucose to relieve the inhibition of glucose utilization than it does of glycerol utilization. This implied that in T. gambiense, D-2-D inhibited the utilization of hexoses and glycerol at least two sites. Further evidence to support the conclusion that two sites exist came from work with glucosamine. Glucosamine, which is not utilized by T. gambiense, is structurally very similar to D-2-D. Both analogues are identical to glucose, except for the loss of the hydroxyl group at carbon number two. Glucosamine was shown to inhibit the utilization of glycerol but had no apparent effect of the utilization of glucose (Figure 4). It should also be noted that increasing the glycerol concentration had no apparent effect on the inhibition of glycerol utilization by D-2-D.

In an attempt to determine the type of chemical structure required to inhibit both the utilization of glucose and glycerol, as well as relieve the inhibition of glycerol utilization, the effect of many compounds structurally similar to glucose were studied. These results are shown in Table 2. It was also determined which of the compounds investigated were capable of being phosphorylated by hexokinase and of being utilized. From the data obtained, it was concluded that inhibitors of both glucose and glycerol fermentation were quite specific. Inhibitors must be aldo-hexoses, which are sufficiently similar to glucose to be capable of being phosphorylated but lack the hydroxyl group at carbon two. Compounds capable of relieving the inhibition of glycerol utilization must also be aldo-hexoses which are capable of being phosphorylated. It would appear that the only alteration which can occur in the glucose molecule is the rotation of the hydroxyl group around carbon two, as in the case of mannose.

Since both D-2-D and glucosamine are capable of being phosphorylated, it is possible that a phosphorylated intermediate was the actual inhibitor of glucose and glycerol utilization. If a metabolic intermediate is the inhibitor, it does not appear to accumulate within the cell. The kinetics of oxygen uptake after the addition of glucose to cells incubated for varying periods of time in glycerol plus D-2-D were identical (Figure 3). Also, since there was an immediate linear stimulation of oxygen uptake after the addition of glycerol to cells utilizing glucose or mannose, it is believed that the enzymes involved in the metabolism of glycerol are constitutive (Figure 5). Therefore, D-2-D does not appear to inhibit the formation of any inducible enzymes necessary for the metabolism of glycerol.

It was found that D-2-D does inhibit carbohydrate (and glycerol) metabolism of T. gambiense in the presence of normal rabbit serum or of trypanosomes present in whole mouse blood altered only by the presence of anticoagulant. Therefore, it is assumed that the inhibition by D-2-D was not an artifact of an unnatural environment.

Also, the inhibitory effects of D-2-D on the metabolism of both T. gambiense and T. rhodesiense would suggest that these results are not due to a peculiarity of a single strain or species of African trypanosomes.

DISCUSSION

2-deoxy-d-glucose was shown to inhibit the fermentation of glucose, mannose, fructose, and glycerol in Trypanosoma gambiense. The fermentation of mannose, fructose, and glycerol, but not glucose, was also inhibited by D-2-D in Trypanosoma rhodesiense. Results similar to those obtained with the trypanosomes have been obtained with yeast (Scharff, T.G., 1961, Scharff, T.G., and Kremer, III, E. H., 1962). In addition, normal and tumor cells have been found to be sensitive to D-2-D (Woodward, G. E., and Hudson, M.T., 1954).

In T. gambiense inhibition(of glucose and glycerol utilization) by D-2-D presumably occurs at two separate sites. Another inhibitor of glycerol fermentation is glucosamine, which is structurally similar to D-2-D, and inhibits only glycerol utilization under the conditions employed. The fact that only compounds which can be phosphorylated are inhibitors suggests two possibilities: (1) that phosphorylated hexose intermediates are the actual inhibitors, or (2) that the hexose itself is the inhibitor but must be structurally capable of being phosphorylated by hexokinase. It is, however, apparent that inhibitors of both hexose and glycerol fermentation are quite specific in their chemical structure.

There is some evidence in the literature to suggest that an active transport mechanism is involved in the uptake of amino acids, galactose and various ions in trypanosomes (Williamson, J., and Desowitz, R.S., 1961, Warren, L. G., and Kitzman, W.B., 1963, and Mulvey, Jr., P.F., 1960). Also, phosphorylated intermediates of both glucose and glycerol metabolism are not utilized by intact cells of T. gambiense and T. rhodesiense, but they are in cell-lysates. In addition, several hexoses which competitively inhibit the phosphorylation of glucose had no effect on glucose utilization in intact cells, even though one of the hexokinase inhibitors (mannose) can be utilized by the African trypanosomes (Seed, J.R., unpublished observations). These data are compatible with the concept of a transport system for carbohydrates in the African trypanosomes. The similarity between the results reported in this paper and those obtained with yeast might, therefore, suggest that D-2-D also inhibits fermentation in trypanosomes by blocking a transport mechanism. The inability to inhibit several enzymes of the hexose or triose portion of the Embden-Meyerhof-Parnas Pathway by D-2-D would support this hypothesis (Seed, J.R., unpublished observations). Grant and Sargent (1960) have shown that glycerophosphate is accumulated in cells respiring in glycerol but not in glucose. It is, therefore, possible to explain the long lag prior to inhibition of glycerol metabolism by D-2-D by assuming that glycerophosphate, but not

glycerol, can be utilized in the presence of D-2-D. If this assumption is true, then D-2-D must inhibit either glycerokinase or a transport system for glycerol.

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Table I

The effect of increased glucose concentrations on the oxygen uptake of *Trypanosoma gambiense* inhibited by D-2-D.

um glucose/ um D-2-D	% inhibition of oxygen uptake ø Exp. 1 glucose fermentation	Exp. 2* glycerol fermentation
5.5	00.0	-
2.5	30.0	-
1.5	78.0	-
1.0	81.0	00.0
0.5	34.0#	28.0
0.25	-	37.0
0.125	-	64.0
0.075	-	69.0
0.00	-	86.0

ø In experiment 1 trypanosomes were incubated in glucose and D-2-D, varying concentrations of glucose were added from the side arm. In experiment 2 the trypanosomes were incubated in glycerol and D-2-D, and varying amounts of glucose added from the side arm.

* Increased concentrations of glycerol had no apparent effect on the per cent inhibition by a constant amount of D-2-D.

\# This is the maximum inhibition obtainable with D-2-D.

Table 2

The effect of various substrates on the metabolism of *Trypanosoma gambiense*

Substrate	Inhibitors of glucose - glycerol utilization		Compounds capable of relieving the inhibition of glycerol utilization	Compounds phosphorylated by hexokinase	Compounds utilized by *T. gambiense*
D(+)glucose	-	-	+b	+c	+d
D(+)mannose	-	-	+	+	+
L(-)fructose	-	-	-	+	+
2-deoxy-d-glucose	+a	+	-	+	-
D-glucosamine	-	+	-	+	-
D(+)galactose	-	-	-	-	-
D-sorbitol	-	-	-	-	-
L(-)sorbose	-	-	-	-	-
methyl-d-glucose	-	-	-		-
2-deoxy-d-ribose	-	-	-	-	-

+a Inhibitory
b Reversal of inhibition by D-2-D
c Phosphorylated by trypanosome extracts
d Utilized by *Trypanosoma gambiense*
- No effect found.

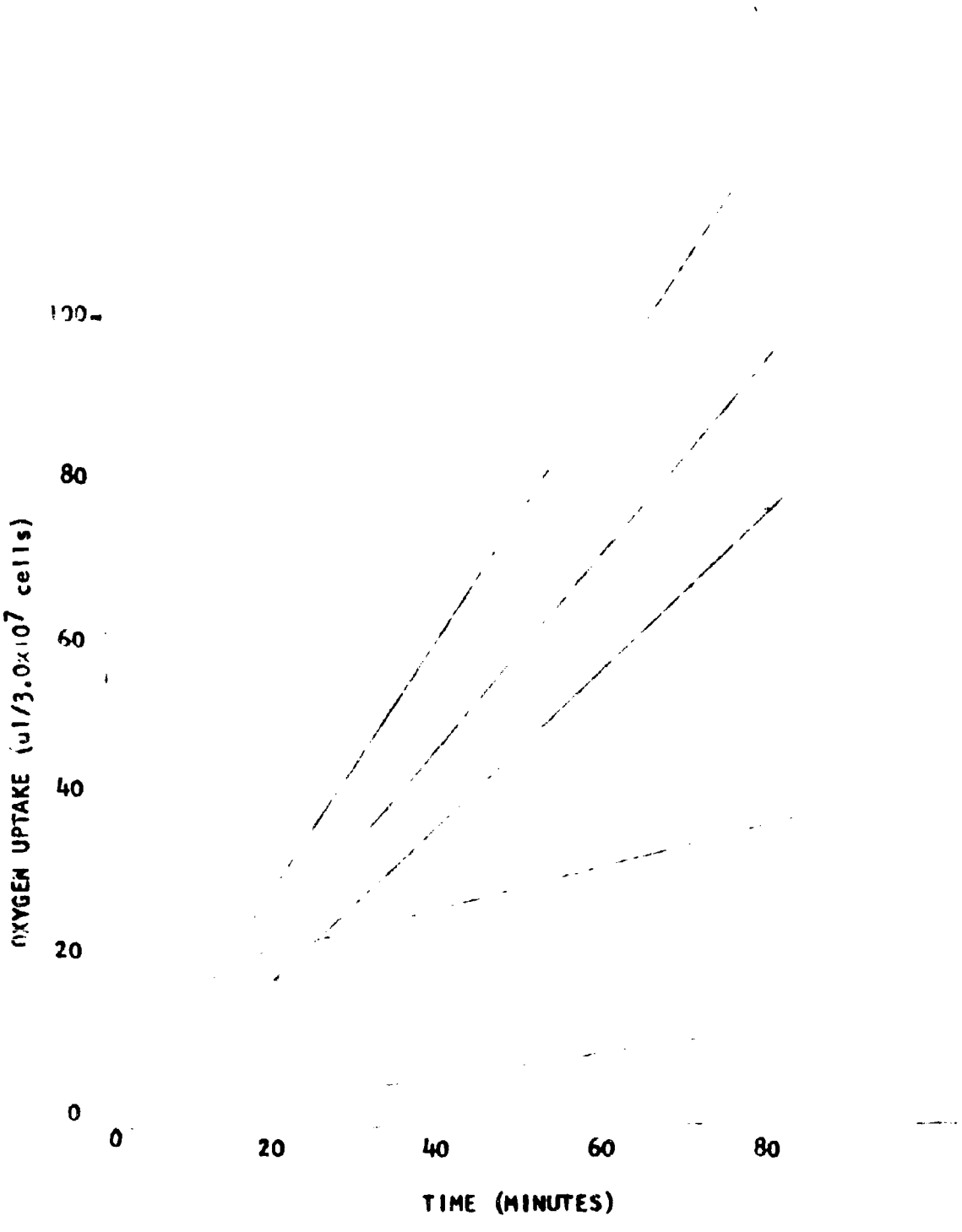

FIGURE 1: The effect of D-2-D on the respiration of *Trypanosoma gambiense* suspended in glucose, mannose, or glycerol. Glycerol (20um). Glucose (20um). Mannose (20um). Glycerol (20um) plus D-2-D (40um). Mannose (20um) plus D-2-D (40um). Glucose (20um) plus D-2-D (40um). D-2-D was added from the sidearm at zero time.

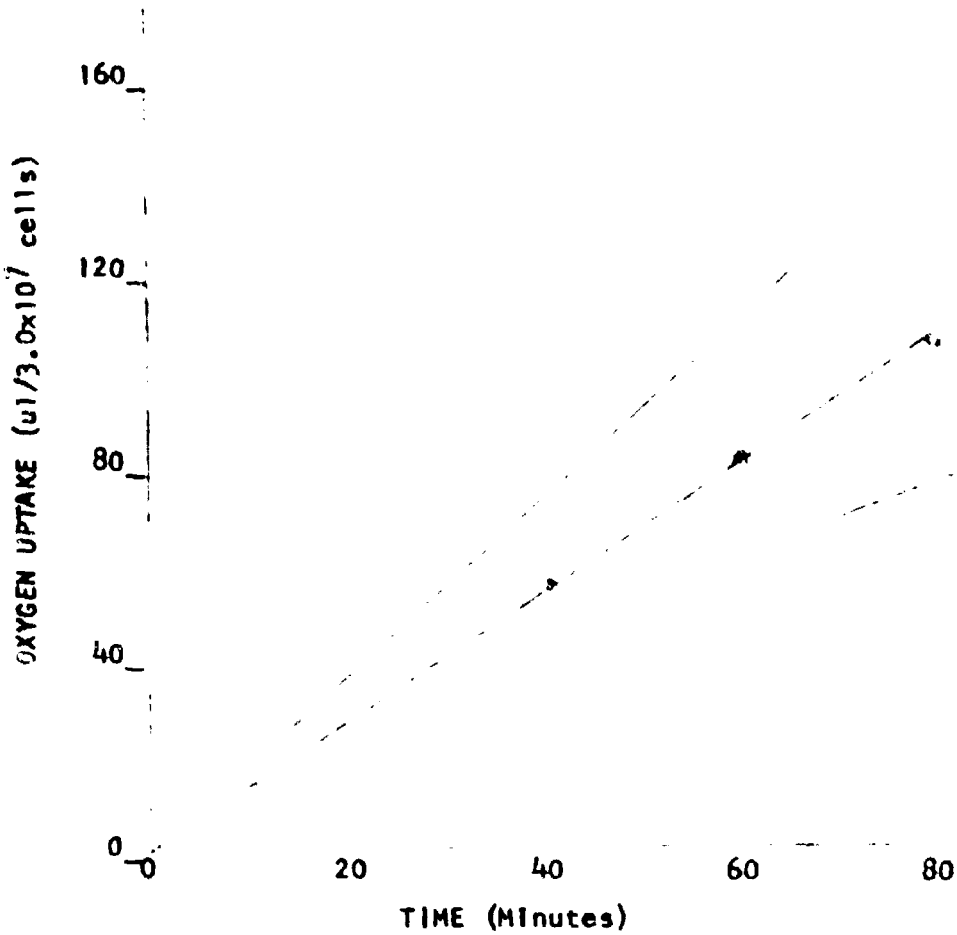

FIGURE 2: The effect of D-2-D on the respiration of *Trypanosoma rhodesiense* suspended in glucose or mannose. Glucose (20um), Glucose (20um) plus D-2-D (40um), Mannose (20um), Mannose (20um) plus D-2-D (40um). D-2-D was added from the side arm at 30 minutes.

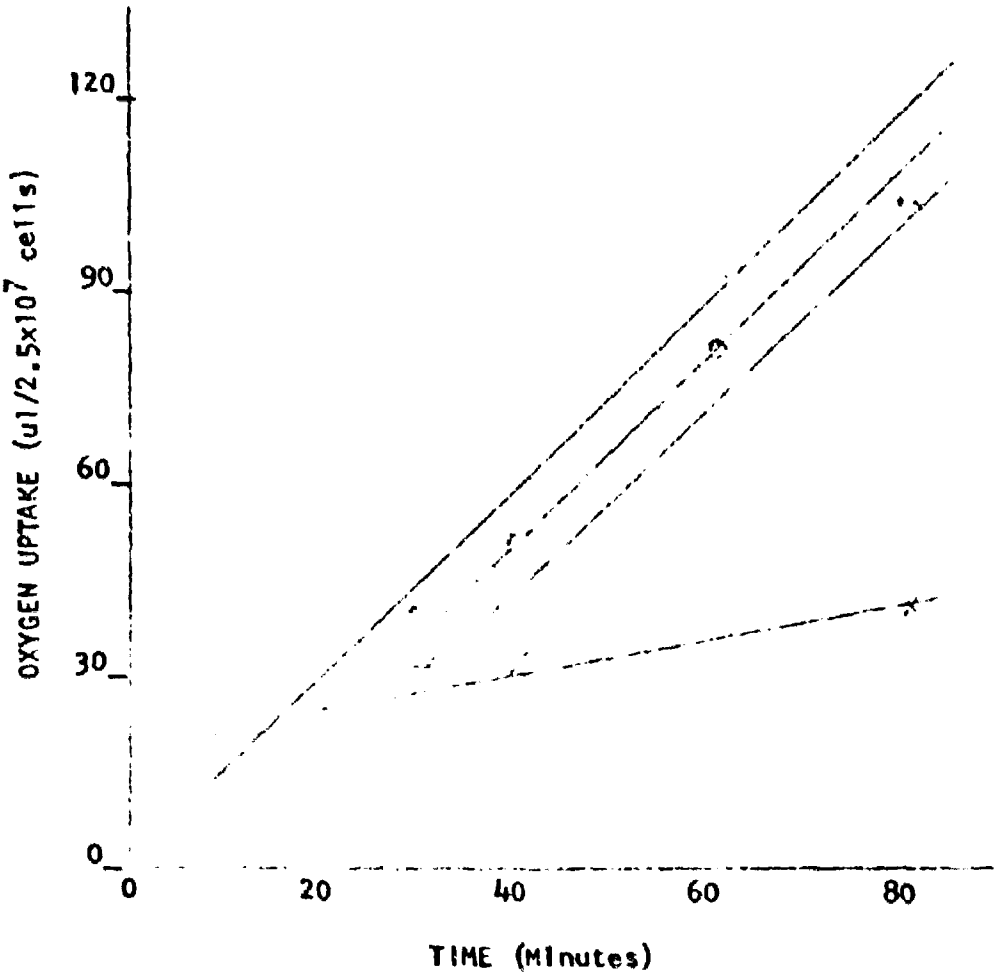

FIGURE 3: The release by glucose of the inhibition of oxygen uptake by D-2-D in *Trypanosoma gambiense*. Glycerol (20um). , Glycerol (20um) and D-2-D (40um) plus glucose (40um) added from the side arm after 10 minutes. Glycerol (20um) and D-2-D (40um) plus glucose added after 20 minutes. Glycerol (20um) and D-2-D (40um). D-2-D was added from the side arm 2 minutes before zero time.

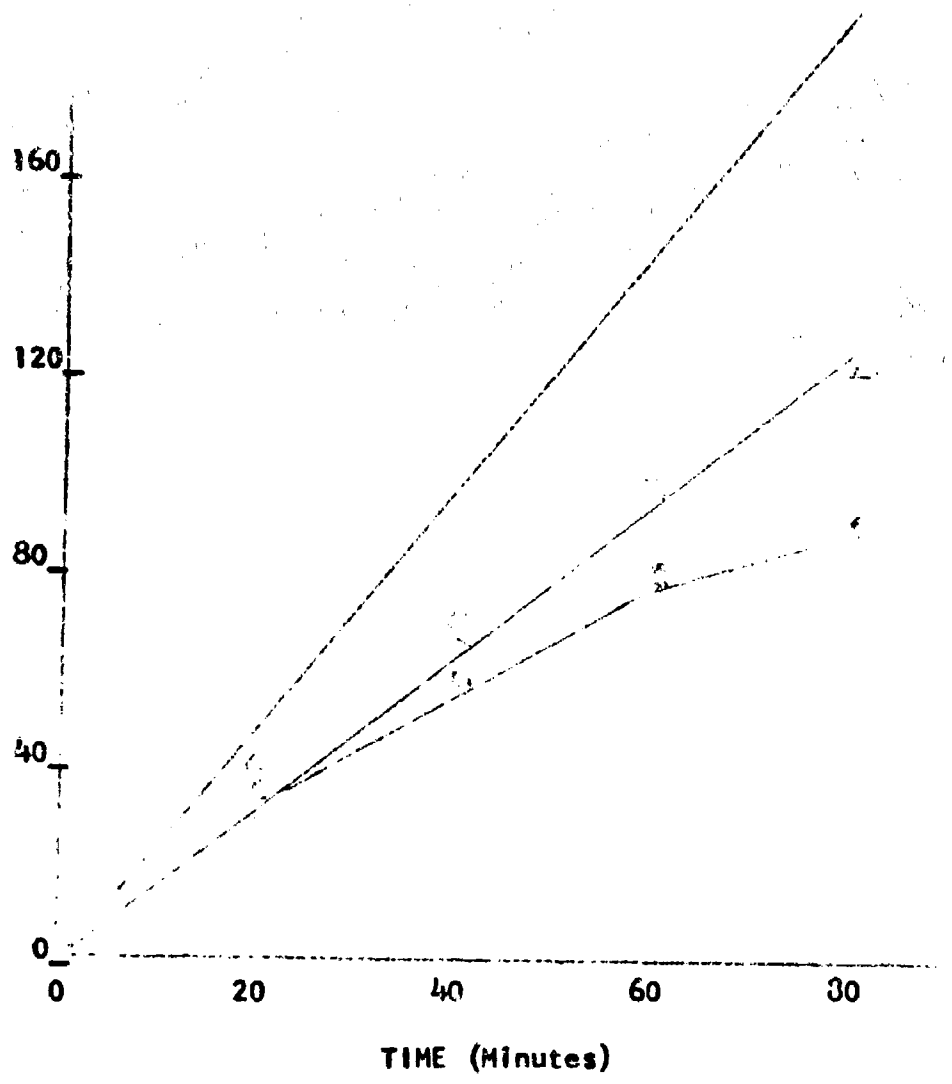

FIGURE 4: The inhibition by glucosamine of oxygen uptake by *Trypanosoma gambiense* in the presence of glycerol. Glycerol (20um) Glucose (20um) plus glucosamine (40um), Glucose (20um), Glycerol (20um) plus glucosamine (40um), Glucosamine added from side arm at zero time.

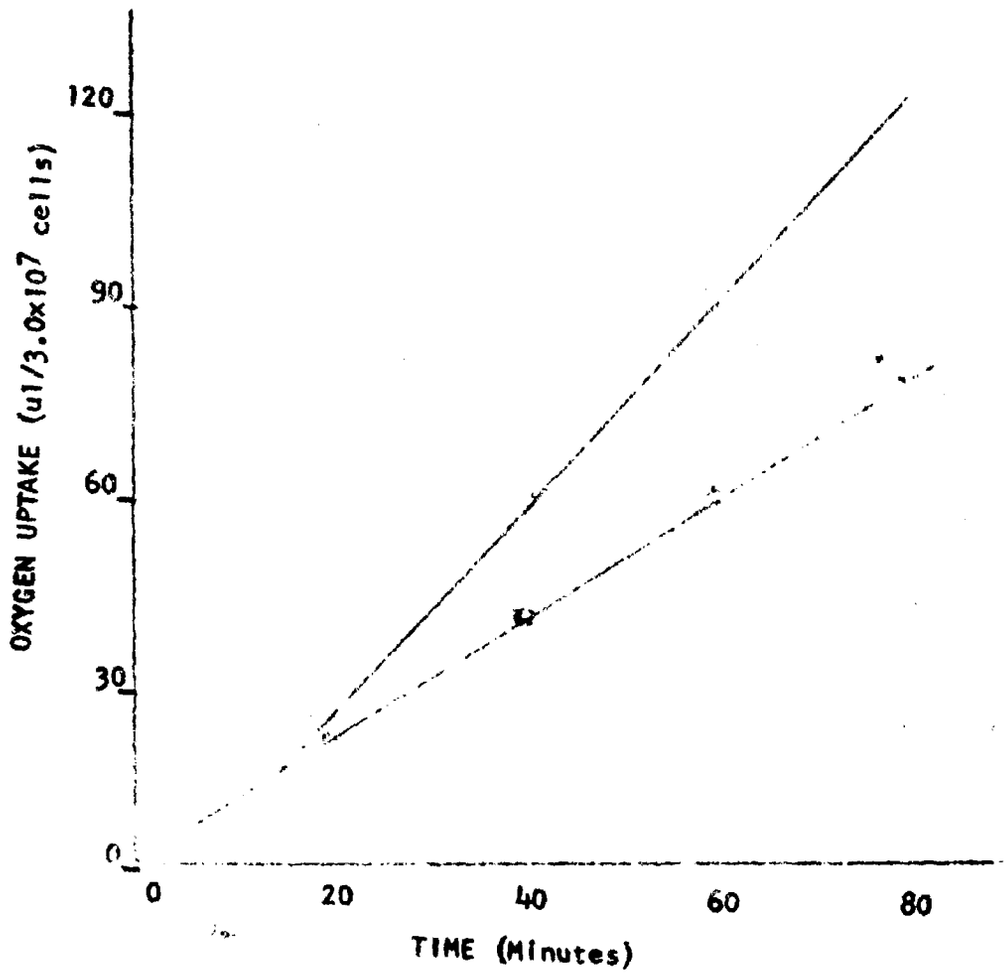

FIGURE 5: The stimulation of oxygen uptake by the addition of glycerol to *Trypanosoma gambiense* suspended in mannose. ○ Mannose (20um) plus glycerol (40um) added from sidearm after 15 minutes. ● Mannose (20um).

ANNUAL PROGRESS REPORT

Project 3A012501A813-02, Communicable Diseases and Immunology-Communicable Diseases

Task 2, The Characterization of Hexokinase from *Trypanosoma rhodesiense* and *Trypanosoma gambiense*.

Reporting Installation:	Sixth US Army Medical Laboratory Fort Baker, California Parasitology Division
Period Covered by Report:	1 July 1963 through 30 June 1964
Principal Investigators:	John R. Seed, 1st Lt, MSC Mauro A. Baquero, Sp-5
Reports Control Symbol:	MEDDH-298
Security Classification:	UNCLASSIFIED

ABSTRACT

Project 3A012501A318-02 — Title: Communicable Diseases and Immunology-Communicable Diseases

Task No. 2 — Title: The Characterization of Hexokinase from *Trypanosoma rhodesiense* and *Trypanosoma gambiense*

Reporting Installation: Sixth US Army Medical Laboratory, Fort Baker, California

Period Covered by Report: 1 July 1963 through 30 June 1964

Authors: John R. Seed, 1st Lt, MSC; Mauro A. Baquero, Sp-5

Reports Control Symbol: MEDDH-233

Security Classification: UNCLASSIFIED

A Hexokinase found in both the soluble and particulate fractions of *Trypanosoma gambiense* extracts has the following characteristics: A) temperature optimum (50-60°C); B) pH optimum 7.0-9.0; C) required Mg^{++} and ATP; D) inhibited by ADP, p-Hydroxymercuribenzoate and also competitively inhibited by mannose, glucosamine and N-acetyl-D-glucosamine; E) phosphorylated glucose (km 2.3×10^{-4}M), fructose (km 1.4×10^{-3}M), mannose (km 2.6×10^{-4}), 2-deoxy-D-glucose, and glucesamine.

This enzyme was clearly distinguished from yeast hexokinase on the basis of its temperature optimum, inhibition by sulfhydryl inhibitors, and immunological specificity. The inhibition by p-hydroxymercuribenzoate would suggest a similarity to the animal hexokinases. It has also been found that this enzyme is not the rate limiting step in carbohydrate metabolism. Therefore, differences in the rate of hexose utilization by *T. gambiense* can not be accounted for by differences in the rate of phosphorylation of various hexoses.

The enzyme from *T. rhodesiense* has not been as completely characterized as the enzyme from *T. gambiense*, however the preliminary results would suggest a close similarity in their properties.

This paper has been submitted to the Office of the Surgeon General for review and clearance for publication. Upon approval, it will be submitted to the Journal of Protozoology for possible publication.

BODY OF REPORT

Project No. 3A012501A313-02 Title: Communicable Diseases and Immunology-Communicable Diseases

Task No. 2 Title: The Characterization of Hexokinase from Trypanosoma rhodesiense and Trypanosoma gambiense

Description:

Glucose, mannose and fructose are utilized at different rates by the African Trypanosomes (16). These hexoses are known to phosphorylated by hexokinase from a wide variety of sources (1). This suggested that the differences in the rates of sugar utilization by trypanosomes might be due to differences in their rate of phosphorylation by hexokinase.

This paper is an attempt to characterize hexokinase from T. rhodesiense and T. gambiense in order to obtain comparative information and also to determine if hexokinase is the rate limiting step in carbohydrate metabolism.

Progress:

MATERIALS AND METHODS

1. General Methods: Both the (XaR) strain of Trypanosoma rhodesiense and the Willcome (TS) strain of Trypanosoma gambiense have been described previously (12,13). These strains were maintained by syringe passage every second day through 20 g female mice. To obtain large quantities of blood trypanosomes, 200-300 g white male rats were infected with 0.25 ml of infected mouse blood at peak parasitemia. The procedures used to isolate the trypanosomes from infected rat blood are outlined in Figure 1.

2. Hexokinase Assay:

(A) Three methods were used to measure hexokinase activity. The first was the method describe by Darrow and Colowick (7). Reactions assayed by this method were analyzed at 560 mu in a Beckman (Model DU) spectrophotometer. The second method, described by Saltman (11), involves the precipitation of hexose phosphates by Ba $(OH)_2$ and $ZnSO_4$. Residual free glucose is then measured by the Somogyi method as described by A.C. Neish (10). A third method involved the measurement of O_2 uptake by trypanosome lysates in the presence of glucose ATP and DPNH. Oxygen uptake was measured in a conventional Warburg apparatus in air at 31°C.

(B) Trypanosome extracts were obtained by disrupting 0.3 ml of packed cells in 1.0 ml of distilled water with the aid of a teflon homogenizer. The resulting suspension was centrifuged at 3600 RPM for

10 minutes in a clinical centrifuge. The supernatant was used as the enzyme extract. Trypanosome lysates for respiratory studies were prepared by suspending 0.3 ml of packed cells in 1.0 ml of distilled water and then incubating in the cold for 15 minutes to allow for lysis. This lysate was used as the enzyme preparation. In one experiment a cell-lysate of T. rhodesiense was sonicated in a Raytheon Oscillator (Model DF 101) for 15 minutes. The resulting lysate was centrifuged and the sediment resuspended in distilled water to its original volume. Both the supernatant and the sediment were used as enzyme preparations.

(C) Protein was determined by the method of Lowry et al. (8).

(D) As the trypanosome extracts were invariably contaminated by rat blood cells (direct counts indicated a maximum of 3 percent contamination by red blood cells and a smaller percentage of white blood cells and platelets), it was necessary to determine if the contaminates were in any way responsible for hexokinase activity in trypanosome extracts. Therefore, rat blood cell extracts (RCE) (primarily red blood cells) were prepared from packed rat blood cells in a manner identical to that used to prepare trypanosome extracts. The RCE, when assayed by the method of Darrow and Colewick, showed no hexokinase activity. Also, rabbit antiserum prepared against RCE, and which agglutinated rat red blood cells at a titer of 1/300, failed to neutralize or reduce hexokinase activity in trypanosome extracts. Thus the hexokinase activity in trypanosome extracts is not due to the presence of hexokinase from contaminating rat blood cells.

3. Neutralization:

(A) Preparation of rabbit anti-yeast hexokinase. Yeast hexokinase (Sigma, Type III) was suspended in distilled water (2mgs/ml) and then mixed with 1 part of normal saline and 2 parts of Freund's adjuvant (Difco, Complete). This mixture was injected subcutaneously in a series of 4 injections over a period of two weeks.

(B) The neutralization test was described previously (12). Briefly, rabbit anti-yeast hexokinase serum (0.5 ml) was added to the enzyme extract (0.5ml). The mixture was incubated at 37°C for 1 hour and then overnight in the cold at 0-4°C. The suspension was centrifuged at 3500 RPM for 10 minutes in a clinical centrifuge and the supernatant assayed for residual enzyme activity. Controls were always run with normal serum.

4. Materials: Adenosine di- and tri- phosphate (ADP,ATP), Glucose 6-phosphate, 2-deoxy-D-glucose 6-phosphate, reduced diphosphopyridine nucleotide (DPNH), p-Hydroxymercuribenzoate (originally believed by Sigma to be p-chloromercuribenzoate), and yeast hexokinase, type III were all obtained from Sigma Chemical Company, St. Louis 13, Missouri. Inosine triphosphate (ITP) was obtained from Mann Research Laboratories, New York 5, New York.

RESULTS

In extracts of Trypanosoma gambiense prepared by extensive homogenization, hexokinase activity was found in the sediment obtained by low speed centrifugation. One experiment, in which a cell-lysate of T. rhodesiense was sonicated for 15 minutes revealed that hexokinase activity was still present in the low speed sediment (12,000xG). Therefore, hexokinase in both T. gambiense and T. rhodesiense is at least partially particulate in nature. Hexokinase activity was also found in the supernatant fraction after centrifugation. It is, however, not known if this was due to the release of hexokinase during preparation or if the enzyme actually exists in the cell in both a soluble and particulate state.

Hexokinase from T. gambiense is capable of phosphorylating glucose, mannose, fructose 2-deoxy-D-glucose and glucosamine but fails to phosphorylate at least 9 other sugars (Table 1). For comparison, hexokinase from T. rhodesiense and yeast were studied and similar findings were obtained with the sugars tested. These results have also been included in Table 1. The results obtained with yeast hexokinase are identical to those obtained by other investigators (4,9).

The relative rates of phosphorylation of the various sugars by trypansome and other hexokinase are shown in Table 2. It can be seen that only the relative rates of phosphorylation of fructose by the various hexokinases appear significantly different. The Michaelis constants for the enzymes from T. rhodesiense, T. gambiense and yeast were determined. These results plus the Michaelis constants for hexokinase from various other sources are included in Table 3.

The effect of adding varying amounts of mannose, glucosamine or N-acetyl-D-glucosamine to a system containing various concentrations of glucose was determined (Table 5). All three hexoses competitively inhibit the phosphorylation of glucose by extracts of T. gambiense. Similar results were found with yeast hexokinase. Mannose has been found to competitively inhibit the phosphorylation of glucose by extracts of T. rhodesiense. The results obtained by testing the effects of various other sugars on the phosphorylation of glucose by T. gambiense are shown in Table 1.

The effects of various co-factors and inhibitors on T. gambiense, yeast, and Euglena hexokinase are shown in Table 4. 2-deoxy-D-glucose apparently had little effect on the phosphorylation of glucose (Figure 2). There was a 12% reduction in the rate of oxygen uptake in the presence of 2-deoxy-D-glucose, however, it is doubtful if this small a reduction is significant.

The temperature optimum for the hexokinase from T. gambiense is 50-60°C (Figure 3). The temperature optimum for yeast hexokinase was found to be 40°C. The trypanosome enzyme is partially denatured by heating at 50°C for 10 minutes and the enzyme from both yeast and

T. gambiense were completely denatured at 50°C. The discrepancy between the temperature optimum and the heat sensitivity for trypanosome hexokinase can be accounted for by assuming that although the enzyme was most active in the 50-60°C range, it was being denatured during the 10 minute incubation period. Therefore the actual or biological temperature optimum is 40°C. At 40°C the enzyme rate approached the maximum rate and the enzyme was not denatured. The enzyme was active over a wide pH range with maximum activity between pH 7.0 and 9.0. The enzyme activity was found to be greater at the same pH in "tris" buffer than in phosphate buffer. There was very little difference in enzyme activity using glycyl-glycine buffer or "tris" buffer at the same pH.

CONCLUSIONS

The location of hexokinase activity in both the soluble and particulate fractions of trypanosome extracts is identical to that found with hexokinase obtained from other organisms (5,11). The temperature optimum of yeast hexokinase and Trypanosoma gambiense hexokinase do differ. However, the biological temperature optimum (the temperature at which maximum activity was found in the absence of enzyme denaturation) was found to be 40°C. The optimal pH for maximum activity was in the pH 7.0 to 9.0 range. These values, temperature optimum and pH range, are similar to those obtained with other hexokinases (1,4 & 11).

Trypanosoma gambiense hexokinase requires ATP. Inosine triphosphate only partially replaces this ATP requirement (Table 4). This is similar to hexokinases obtained from higher plants but is different from that found with Euglena hexokinase (1). Trypanosoma gambiense also requires a divalent metal co-factor. In the absence of Mg^{++} little enzyme activity was found. Cobalt, which restored 37% of the activity of Euglena hexokinase, failed to significantly restore the enzyme activity of T. gambiense hexokinase. Calcium, as with Euglena hexokinase, inhibited the hexokinase activity from T. gambiense (Table 4).

The Km values obtained for trypanosome hexokinase appear similar to those obtained for yeast, plant, and Euglena, but differed from that obtained for brain hexokinase (Table 3).

A sulfhydryl inhibitor, p-Hydroxymercuribenzoate, inhibited hexokinase from T. gambiense but had little or no effect on yeast hexokinase (Table 4). The hexokinase from T. equiperdum has also been shown to be inhibited by sulfhydryl inhibitors (3). Trypanosome, muscle, and brain hexokinase appear to be sulfhydryl enzymes, whereas yeast, higher plants, and Euglena hexokinase are apparently not (1,6, & 11). This would suggest, in contrast to the Km values, a closer similarity of the trypanosome enzyme to animal hexokinases.

Adenesine diphosphate inhibits trypanosome hexokinase activity.

This is identical to the results obtained with all hexokinases previously investigated. Glucose 6-phosphate and 2-deoxy-D-glucose 6-phosphate failed to inhibit T. gambiense hexokinase (Table 4). This is in contrast to animal hexokinases in which glucose 6-phosphate is known to inhibit hexokinase activity (5). The sugar 2-deoxy-D-glucose does not appear to significantly inhibit hexokinase activity (Figure 2). This sugar was phosphorylated by trypanosome hexokinase at the same rate as glucose. This is similar to the results obtained with brain hexokinase (6). It should be noted that in order to obtain oxygen uptake by cell-lysates of T. gambiense in the presence of glucose at least six enzymes of the Embden-Meyerhof-Parnas pathway must be active; hexokinase; phosphohexoisomerase; phosphohexokinase; aldolase; L-α-glycerophosphate dehydrogenase; and L-α-glycerophosphate oxidase. This would suggest that the inhibition of glucose utilization by 2-deoxy-D-glucose with intact trypanosomes (13) is not due to the inhibition of any one of these enzymes.

Mannose, glucosamine, and N-acetyl-D-glucosamine competitively inhibited the phosphorylation of glucose (Table 5). This is similar to results obtained with yeast (see Table 4) and brain hexokinase (6,14). Xylose which is known to competitively inhibit brain hexokinase (15) had no apparent effect on either yeast or trypanosome hexokinase (Table 1). None of the competitive inhibitors appear to have any effect on glucose utilization by intact cells. This would suggest a selective permeability to hexoses.

Comparison has been made of the relative rates of utilization of glucose, mannose and fructose by intact cells with the relative rates of phosphorylation of these hexoses by extracts of T. gambiense (See Table 6). It is reasoned that if hexokinase was the rate limiting step in the metabolism of hexoses then the rates of utilization should be identical to the rates of phosphorylation. Since the rates are not identical, the rate limiting steps must be either before or after hexokinase. Further evidence to eliminate hexokinase as the rate limiting step was obtained by comparing the micromoles of glucose utilized by a specific number of cells (mg protein/ml) with the micromoles of glucose capable of being phosphorylated by a cell extract prepared from an equivalent number of cells. As noted in Table 7, the cells are capable of phosphorylating almost three times more glucose than they utilize. This data further suggests that hexokinase could not be the rate limiting step in glucose metabolism. Therefore, the differences in the rate of utilization of glucose, mannose and fructose are not due to the differences in the relative rates of phosphorylation of these hexoses.

Previous work (13) suggested that an active transport system is involved in carbohydrate utilization. This suggestion is strengthened by: 1.) The inability of 2-deoxy-D-glucose to inhibit enzymes involved in glucose utilization (hexokinase, phosphohexoisomerase, phosphohexokinase, aldolase, L-α-glycerophosphate dehydrogenase, and L-α-glycerophosphate oxidase) by cell-lysates; 2.) The apparent selective

permeability of the trypanosomes to hexoses; and, 3.) The inability to account for the differences in rates of utilization of the various hexoses by the differences in their rates of phosphorylation. Since the majority of enzyme steps after hexokinases are identical, the rates of utilization of the various hexoses should be equal if one of these later steps was rate limiting. Therefore, this would suggest a step prior to hexokinase as being rate limiting and this step presumably would be one involved in the transport of hexoses.

It was found that trypanosome hexokinase was not inhibited by rabbit anti-yeast hexokinase, nor did trypanosome extracts absorb out anti-yeast hexokinase antibodies. An antigenic similarity between hexokinases from blood and culture forms of T. rhodesiense has been demonstrated (12). Therefore, although yeast and trypanosome hexokinases have many characteristics in common, they are dissimilar enzymes. This is supported by various other differences found between the two enzymes; i.e., sensitivity to p-Hydroxymercuribenzoate, and the temperature optimum. It is hoped that by comparing the immunological and chemical characteristics of hexokinases from other flagellates and trypanosomes that a better understanding of the evolution of the family Trypanosomidae may eventually be obtained.

Rats infected with trypanosomes.

↓

B'ed using 2.5% sodium citrate as the anticoagulant.
1 part anticoagulant to 5 parts of whole blood.

↓

Centrifuged at 1300 RPM for minutes in
an International Clinical Centrifuge. → RBCs discarded

↓

Centrifuged at 1900 RPM for 4 minutes.

↓

Blood trypanosome (ediment) resuspended in saline
(10 volumes diluent).

↓

(Recentrifuged at 1900 RPM and then resuspended)
(in fresh saline.) → Supernatants discarded

↓

Recentrifuged at 2300 RPM, supernatant discarded
and cells stored at -30°C in a Revco deep freezer
until needed.

Procedure used for isolating blood trypanosomes.
Additional trypanosomes can be obtained by re-
suspending the discarded RBCs in saline and
repeating the entire isolation procedure.

FIGURE 1

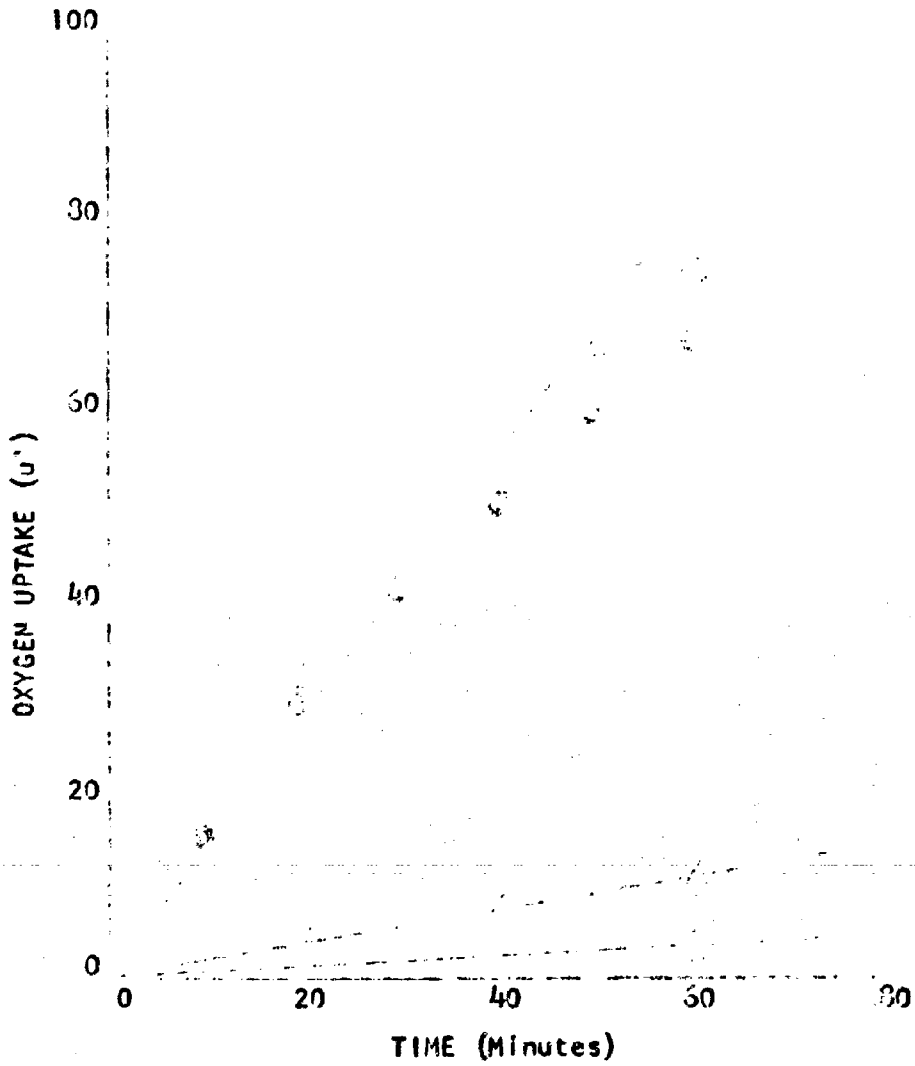

FIGURE 2: The effect of 2-Deoxy-D-glucose (D-2-D) on the respiration of cell-lysates of *Trypanosoma cambiense*. O Endogenous; X glucose (40um), and DPNH (3um); △ glucose (40um), DPNH (3um), and ATP (20um); ● glucose (40um), DPNH (3um), ATP (20um), and D-2-D (40um).

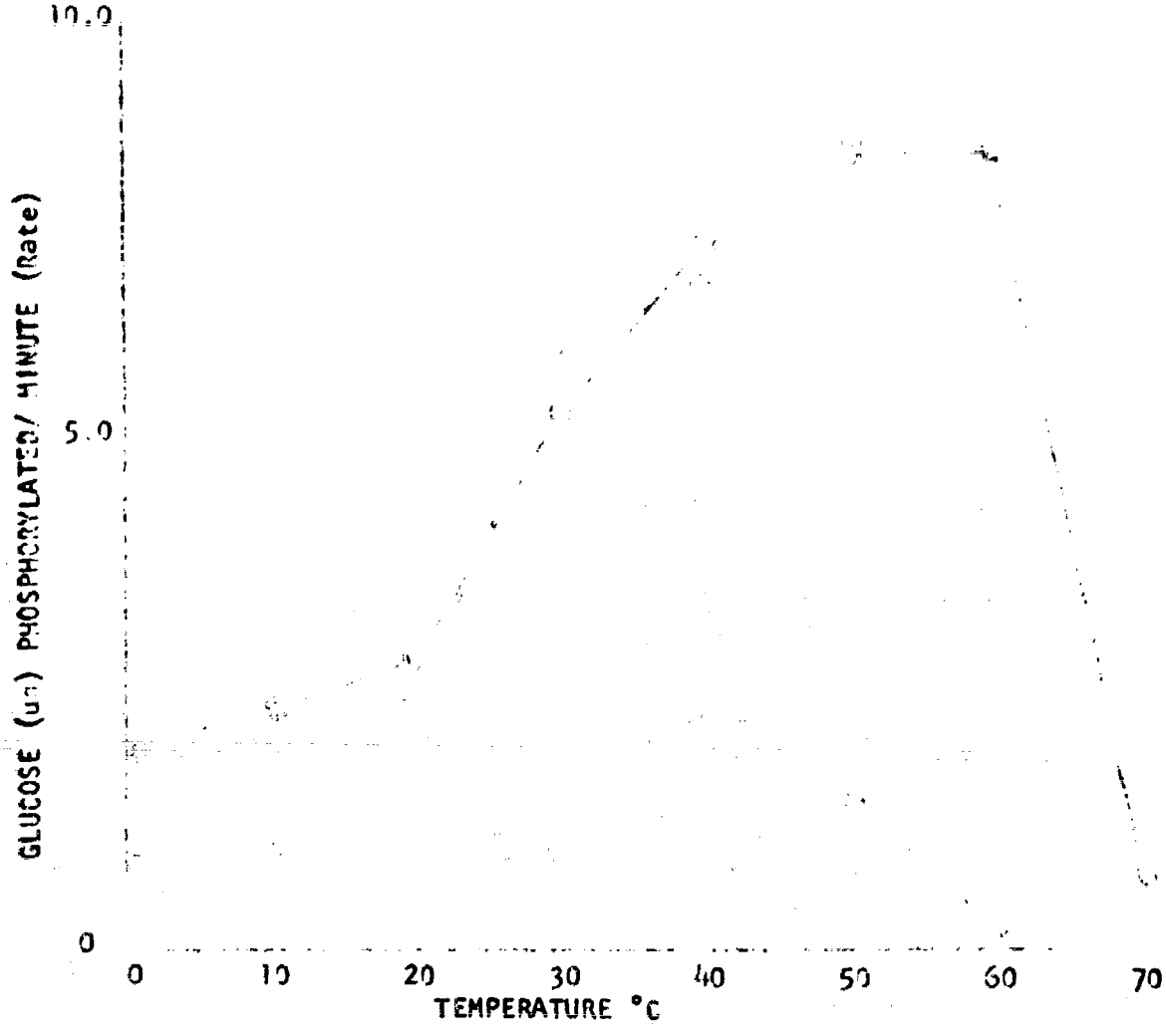

FIGURE 3: The effect of temperature on activity of Trypanosoma gambiense (O) and yeast hexokinase (X). Hexokinase activity was measured by method of Saltman (11). Enzyme (0.1 ml suspended in distilled water) was incubated with "tris" buffer (0.1M), pH 8.5 (0.3 ml) and hexokinase reagent (0.8 ml) for 10 minutes. Hexokinase reagent contained ATP (0.02M), Glucose (0.01M), $MgCl_2$ (0.1M), and NaF (0.001M) and was neutralized to pH 7.0 with NaOH. Activity was measured by sugar disappearance.

TABLE 1

Compounds phosphorylated by hexokinase from *T. rhodesiense*, *T. gambiense* and yeast

Substrate	Compounds phosphorylated by hexokinase from: T. gambiense	T. rhodesiense	Yeast
D(+) glucose	+	+	+
D(+) mannose	+(I)[a]	+(I)	+(I)
L(-) fructose	+(0)[a]	+(0)	+(0)
2-deoxy-D-glucose	+(NI)[a]	+(0)	+(0)
D-glucosamine	+(I)[a]	0	+(I)
N-acetyl-D-glucosamine	-(I)	0	-(I)
D(+) galactose	-(NI)[a]	-(0)	-(NI)
D- sorbitol	-(NI)[a]	0	-(NI)
L(-) sorbose	-(NI)[a]	0	-(NI)
α-methyl-D-glucose	-(NI)[a]	0	-(NI)
Arabinase	0	-(0)	-(NI)[b]
D(+) maltose	0	-(0)	-(NI)[b]
D(+) Xylose	-(NI)	0	-(NI)
2-deoxy-D-ribose	-(NI)[a]	0	-(NI)

+ Compound phosphorylated.
- Compound not phosphorylated.
(I) Compound competitively inhibited hexokinase activity.
(NI) Compound failed to competitively inhibit hexokinase activity.
0 Compounds not tested as hexokinase substrates.
(0) Compounds not tested as a competitive inhibitor of hexokinase.
a Seed, J.R. (13).
b McDonald, M.R. (9).
Assays performed by method of Darrow and Colowick (7).

TABLE 2

Comparative Rates of Hexokinase Activity

Enzyme Source	Glucose	Fructose	Mannose	D-2-D[g]	Glucosamine	References
T. rhodesiense	1.00	-	0.54	1.00	-	-
T. gambiense	1.00	1.00	0.50	1.00	0.34	-
Yeast	1.00	1.24	0.43	0.94	0.52	-
Yeast	1.00	1.40	0.30	-	-	(2)
Euglena	1.00	1.19	0.55	-	-	(1)
Higher Plants	1.00	0.52	0.53	-	0.52	(11)
Muscle	1.00	1.30	0.30	-	-	(4)
Brain	1.00	1.50	0.40	1.0	-	(15)

g D-2-D 2-deoxy-D-glucose.
Assays performed by the method of Darrow and Colowick (7).

TABLE 3

Km Values For Hexokinases From Various Sources

Enzyme Source	Km Glucose	Km Fructose	Km Mannose	References
T. rhodesiense	4.0×10^{-4} M	-	3.0×10^{-4} M	-
T. gambiense	2.3×10^{-4} M	1.4×10^{-3} M	2.6×10^{-4} M	-
Yeast	4.4×10^{-4} M	1.0×10^{-3} M	2.2×10^{-4} M	-
Yeast	1.6×10^{-4} M	1.7×10^{-3} M	1.0×10^{-4} M	(14)
Brain	8.0×10^{-5} M	1.6×10^{-3} M	5.0×10^{-5} M	(15)
Higher Plants	4.4×10^{-4} M	-	-	(11)
Euglena	5.0×10^{-4} M	-	-	(1)

Assay performed by method of Darrow and Colowick (7).

TABLE 4

Effect of Various Co-Factors and Inhibitors on Hexokinase From *T. Gambiense*, Yeast and Euglena

	Percentage Hexokinase Activity		
	Yeast	T. gambiense	Euglena*
Complete System (CS)	100	100	100
CS Minus Mg^{++}	-	20	7
CS Minus ATP	-	0	0
Co^{++} replacing Mg^{++}	-	1	37
ITP replacing ATP	-	58	3
CS plus ADP	62	82	79
CS plus Glucose 6-phosphate	-	100	-
CS plus Ca^{++}	-	52	Inhibitory
CS plus p-Hydrosy-mercuribenzoate	92	30	33

* Belsky & Schultz (1)
Assay performed by method of Darrow and Colowick (7)

TABLE 5

Effect of Mannose, Glucosamine and N-Acetyl-D-Glucosamine (N-A-G) on Hexokinase Activity

um Glucose*	um Mannose	um Glucosamine	um N-A-G	% Activity
40	0	-	-	100
30	10	-	-	89
20	20	-	-	85
10	30	-	-	77
0	40	-	-	58
40	-	0	-	100
30	-	10	-	73
20	-	20	-	64
10	-	30	-	50
0	-	40	-	34
40	-	-	0	100
-	-	-	-	-
20	-	-	40	73
10	-	-	40	27
0	0	-	40	0

* Assay performed by method of Darrow & Colowick (7).
In all experiments, except these in which glucose was not present, saturating concentration of glucose were employed (10 um.= 3.5x10-3M).

TABLE 5

The Relative Rates of Utilization and Phosphorylation of Various Hexoses

Relative Rates of:	Glycerol	Fructose	Mannose	Glucose
		T. gambiense Substrate		
ø Utilization	1.4	1.2	0.9	1.0
* Phosphorylation	-	1.0	0.5	1.0
		T. rhodesiense		
Utilization	1.4	-	0.85	1.0
Phosphorylation	-	-	0.54	1.0

ø Determined by oxygen uptake (13)

* Assay performed by method of Darrow and Colowick (7)

TABLE 7

The Micromoles of Substrate Utilized Compared with the Micromoles of Substrate Phosphorylated by *T. gambiense*

	Glucose	Substrate Mannose	Fructose
ø Utilization (um)	6.04	7.14	7.37
* Phosphorylation (um)	21.70	1[illegible].02+	21.70+

ø Determined by oxygen uptake (13). These values are based on the assumption that approximately one um of substrate is utilized per one um of oxygen taken up.

* Assayed by method of Darrow and Colowick (7).

+ The figures given for the um of mannose and fructose phosphorylated are calculated values. The calculations are based on relative rates of phosphorylation; i.e., Mannose is phosphorylated at 50% the rate of glucose.

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ANNUAL PROGRESS REPORT

Project 6X6-01-001-01, Preventive Medicine - Communicable Diseases

Task 3, Studies on Sulfonamide Resistance of Neisseria Meningitidis

Reporting Installation:	Sixth US Army Medical Laboratory Fort Baker, California
Period Covered by Report:	1 December 1963 through 30 November 1964
Principal Investigators:	Adrian D. Mandel, Lt Col, MSC Donald K. Hesson, Maj, MSC
Reports Control Symbol:	MEDDH-288
Security Classification:	UNCLASSIFIED

ABSTRACT

Project 6X6-01-001-01 — Title: Preventive Medicine - Communicable Diseases

Task No. 3 — Title: Studies on Sulfonamide Resistance on Neisseria Meningitidis

Reporting Installation: Sixth US Army Medical Laboratory, Fort Baker, California

Period Covered by Report: 1 December 1963 through 30 November 1964

Authors: Adrian D. Mandel, Lt Col, MSC; Donald K. Hesson, Major, MSC

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Strains of N. meningitidis were obtained from a representative population of soldiers at a number of military posts in the Sixth US Army area. One post, experiencing a meningococcal epidemic, recorded carrier rates ranging from 15% to 72%, while three other installations, free of meningococcal infections for at least 2 years prior to sampling, recorded carrier rates of 15%, 40%, 31%. As many strains as possible were examined for serological identity and resistance to sulfadiazine. Over 90% of the strains from all sources were type "B", the remainder of the strains were type "C" or "A", plus a number of strains that could not be typed. Sulfa-resistance was determined at the 0.1 mg % level. At the meningitis-free posts, the percentages of strains resistant to sulfa were 0, 15, 12, while at the epidemic post the values ranged from 6 - 81. A comparison of the carrier rate in companies experiencing meningitis and in companies free of meningococcal infections failed to demonstrate any correlation. The most suitable method for the storage of meningococcal cultures was on blood agar slants covered with sterile mineral oil.

BODY OF REPORT

Project 6X6-01-001-01 Title: Preventive Medicine - Communicable Diseases

Task No. 3 Title: Studies on Sulfonamide Resistance on Neisseria Meningitidis

Description:

The objectives of this study are: (1) To determine the meningococcal carrier rate at a number of Army installations during a non-epidemic period and at an Army post experiencing a meningococcal epidemic. (2) To determine the serological type and sulfa resistance of the isolated strains. (3) To preserve these strains by lyophilization.

Progress:

Studies on sulfonamide resistance of N. meningitidis.

a. The medium used in these studies for the isolation of N. meningitidis was that of Thayer and Martin (Pub. Hlth Reports 79; 49, 1964). This medium is a Mueller Hinton base containing 25 units/ml of Polymyxin B and 10 mcg/ml of ristocetin and is superbly suited for the recovery of N. meningitidis from the nasopharynx. T-M medium permits luxuriant characteristic growth of meningococci after 18 - 24 hours at 37°, while almost completely inhibiting all other bacteria normally present in a nasopharynx. In many instances, it is possible to type meningococci directly from the T-M plates. Fermentation reactions were determined on a phenol red agar base with added 1% carbohydrate and 5% inactivated human serum. Serological typings were performed with Difco antiserum. For typings, the organisms were suspended in saline on a slide and mixed with antiserum.

b. The determination of resistance to sulfadiazine presented difficulties early in the studies. Although Mueller Hinton medium with varying concentrations of sulfadiazine was used for the determination of sulfa resistance, erratic results were obtained in several instances when the determinations were repeated. Subsequent studies determined that the concentration of inoculum was critical for reproduceable results and that heavy inocula tended to give a false picture of increased resistance to sulfadiazine.

At the suggestion of Lt Col Donald Hunter of WRAIR, all sulfa-resistance determinations were performed by placing a 2 mm loop of organisms, equal in turbidity to a 1:1000 dilution of a number 3 MacFarlane nephalometer, on Mueller Hinton plates containing varying concentrations of sulfadiazine. Table I illustrates the effect of concentration of organisms on the end point of sulfa-resistance.

TABLE I

INOCULUM CONCENTRATION AND END POINT OF SULFA-RESISTANCE

N. Meningitidis #5981

	Sulfa Concentration mg %							
	0	0.1	1	2	5	10	20	40
MacFarlane #3	+	+	+	+	+	+	+	+
1:1000 MacFarlane #3	+	+	+	+	+	-	-	-

c. Due to the inability of the meningococcus to remain viable more than a few days or to survive more than about six successive transfers of laboratory media, it was necessary to find a convenient method for preserving cultures and one that would permit their immediate use in any procedure. After a number of unsatisfactory trials with various holding media, it was found that cultures could be preserved at room temperature for several months if grown on blood agar slants and covered with sterile mineral oil. In this manner, it was possible to keep several hundred cultures available for immediate use without resorting to lyophilization. None of the serological, biochemical, or sulfa-resistance characteristics of any organism examined was changed by this method of storage.

Summary and Conclusion:

A comparison of meningococcus carrier rates at three military installations, which have not recorded any cases of meningococcal meningitis for at least three years, range from 15% to 40%. This indicates that carrier rates in the "normal" military population are somewhat higher than the commonly quoted figure of 5% to 15%. At Fort Ord, where about 50 cases of meningococcal meningitis occurred during this study, carrier rates of companies with meningococcal infections and infection-free companies were compared. No correlation between case rates and carrier rates could be found. Carrier rates do not appear to be of any value in predicting the imminence of conditions favorable for a meningococcal epidemic. The only consistent observation obtained in this study was that a much higher ratio of sulfa-resistant organisms was noted at Fort Ord than at those meningitis-free posts. This may be due to the fact that the previous year a regime of sulfa prophylaxis had been in effect at Fort Ord. The serologic type "B" meningococcus was the predominant organism encountered in this study and was the only serologic type found to be sulfa-resistant. These results are summarized in TABLE II.

These studies will be continued with emphasis placed on the dynamics of the carrier rate.

TABLE II

SITE NASOPHARYNGEAL SWABS TAKEN	DATE	NUMBER IN SAMPLE	STATUS OF SUBJECTS	NUMBER POSITIVE FOR N. MENINGITIDIS	NO. RESISTANT TO 0.1 mg % SULFADIAZINE	SEROLOGICAL TYPES OF N. MENINGITIDIS * A	B	C	D
Ft Baker, Calif.	19 Dec 63	93	Seasoned soldiers Civilian employees	14 (15%)	0/14 (0%)	2	5	6	0
Presidio of S.F.	3 Feb 64	103	Seasoned soldiers	47 (40%)	7/45 (15%)	7	18	12	2
Ft Ord, Calif.	3 Feb 64	109	Recruits, 1st day in Army	22 (20%)	1/18 (6%)	4	10	0	0
Ft Ord, Calif.	6 Feb 64	109	Same men as above, 3rd day in Army	17 (15%)	1/14 (7%)	5	10	0	1
Ft Ord, Calif.	3 Mar 64	97	Same men as above, 30 days in Army	27 (25%)	6/24 (25%)	1	9	9	0
Ft Ord, Calif.	18 Mar 64	195	7th week; experienced 2 cases meningitis in 6th week.	95 (49%)	29/87 (33%)	3	76	12	1
Ft Ord, Calif.	26 Mar 64	212	7th week; no cases meningitis	126 (59%)	94/110 (85%)	1	106	1	1
Ft Irwin, Calif.	7 Apr 64	120	Seasoned soldiers	38 (31%)	4/33 (12%)	1	29	1	0
Ft Ord, Calif.	3 Apr 64	119	7th week basic received at other military post	49 (41%)	34/48 (70%)	0	41	3	0
Ft Ord, Calif.	20 Apr 64	99	7th week recruits included in another report (Surveillance Report).	63 (64%)	50/61 (81%)	2	57	1	0

*Difference between total number positive for N. meningitidis (column 4) and number of cultures typed (column 6) represents serologically non-typable culture.

TABLE II, cont'd

SITE NASOPHARYNGEAL SWABS TAKEN	DATE	NUMBER IN SAMPLE	STATUS OF SUBJECTS	NUMBER POSITIVE FOR N. MENINGITIDIS		NO. RESISTANT TO 0.1 mg % SULFADIAZINE		SEROLOGICAL TYPES OF N. MENINGITIDIS *			
								A	B	C	D
Ft Ord, Calif.	20 Apr 64	8	Cadre of above recruits	4	(50%)	1/4	(25%)	0	1	0	0
Ft Ord, Calif.	27 Apr 64	107	8th week recruits. 4 cases meningitis in previous 2 weeks.	77	(72%)	52/64	(81%)	0	57	8	0
Ft Ord, Calif.	24 May 64	100	7th week recruits 2d surveillance report	46	(46%)	21/45	(47%)	0	39	0	0
Ft Ord, Calif.	8 Jun 64	101	6th week recruits 4 cases meningitis 2 previous weeks	27	(26%)	19/28	(68%)	1	18	1	0
Ft Ord, Calif.	10 Jul 64	99	7th week recruits 4 cases meningitis 18 Jun - 5 Jul	37	(37%)	13/37	(35%)	0	31	0	1
Ft Ord, Calif.	10 Jul 64	17	cadre for above group	7	(41%)	3/7	(43%)	0	5	0	0
Ft Ord, Calif.	15 Jul 64	100	7th week recruits 3rd survey	37	(37%)	7/37	(20%)	0	28	2	0
Ft Ord, Calif.	15 Jul 64	12	Cadre for above group	2	(17%)	0/2	(0%)	0	2	0	0
Ft Huachuca, Ariz.	7 Aug 64	119	Seasoned troops	25	(21%)	0/25	(0%)	0	20	0	0

* Difference between total number positive for N. meningitidis (column 4) and number of cultures typed (column 6) represents serologically non-typable culture.

TABLE 11, cont'd

SITE NASOPHARYNGEAL SWABS TAKEN	DATE	NUMBER IN SAMPLE	STATUS OF SUBJECTS	NUMBER POSITIVE FOR N. MENINGITIDIS		NO. RESISTANT TO 0.1 mg % SULFADIAZINE		SEROLOGICAL TYPES OF N. MENINGITIDIS *			
								A	B	C	[illegible]
Ft Ord, Calif.	2 Sep 64	100	7th week recruits 4th survey	16	(16%)	2/14	(14%)	0	7	5	0
Ft Ord, Calif.	2 Sep 64	10	Cadre for above group	3	(33%)	1/3	(56%)	0	2	0	0
Ft Lewis, Wash.	21 Sep 64	120	Seasoned troops	44	(37%)	16/44	(36%)	0	35	2	0

* Difference between total number positive for N. meningitidis (column 4) and number of cultures typed (column 6) represents serologically non-typable culture.

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