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TITLE: RAPID IDENTIFICATION BY CIRCULAR INTENSITY DIFFERENTIAL SCATTERING

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RAPID MICROBIAL IDENTIFICATION BY CIRCULAR INTENSITY DIFFERENTIAL SCATTERING

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As one of the last platform speakers in this fascinating Conference I have the opportunity to summarize what we have heard during the past few days as a background for my own presentation. I would like to begin by asking a rhetorical question: "Who cares about rapid methods of microbial identification anyway?"

We have heard several answers to that question during this Conference but one of the best is that implied by the chairman of this session, Dr. Isenberg, when he said, at the previous Conference on Rapid Methods: "It is quite proper to question the relevance of clinical microbiology when the clinician must rely on an educated guess...to intercede

effectively during the crucial early hours of disease manifestations."[1] In a similar vein ,just last year Drs. Tilton and Balow alluded: "To the day when clinical microbiology will be a primary diagnostic service rather than a confirmatory one, and when clinical microbiologists and infectious disease specialists can point to significant reductions in morbidity and mortality."[2]

In addition to the medical and humanitarian interest in reducing morbidity and mortality, there is the very real economic impact of rapid microbial identification. In the context of the DRGs (diagnostically related groups) in the U.S., and similar cost-containment measures elsewhere, this becomes important as well.

The economic impact does not arise from the modest part that microbial identification plays in the cost of illness resulting from infectious disease, since this is rarely more than 10% of the total cost. Rather rapid identification has an economic impact because it reduces hospital stays and, simultaneously, reduces intravenous fluids and antimicrobials and time spent in isolation rooms.

The best relevant data I know of comes from Dr. Marilyn Menegus and her colleagues at Strong Memorial Hospital in Rochester, New York and deals with the readmission of neonates to the hospital in their first month of life[3] (and personal communication from Dr. Menegus, March, 1984). Year-round, about 45% of these readmissions are due to suspected sepsis; this figure rises to 70% during the summer months. These children are routinely treated as if they had bacter-

ial disease, that is, they are given intravenous fluids and antimicrobials until bacterial cultures are shown to be negative.

As you might suspect, about 70% of the summer admissions are due to nonpolio enteroviruses. In one group of 33 children, only one had a bacterial infection, 20 had isolatable virus and 12 had presumed viral infections, probably many of the latter were infections due to rotaviruses that can't be cultivated. The important point is that those children in whom a virus was identified in 2 days or less had a shorter hospital stay than those children in whom isolation required 3 days or more - 2.7 versus 3.8 days, respectively, a statistically significant difference - and corresponding reductions in intravenous fluids, isolation charges, and so on. So rapid microbial identification does matter, for financial as well as medical and humanitarian reasons, as I'm sure you'll all agree.

May I have the first slide, please. Here is a tabulation of what seem to me to be the best established rapid methods of microbial identification. The top three methods, coagglutination, counterimmunoelectrophoresis, and fluorescent antibody staining, all require about an hour to perform and require more than 10^5 organisms per ml - how much more is a matter of some controversy.

ELISA, especially when coupled with the avidin/biotin probe, requires 4-6 hours and as few as 10^3 organisms per ml. The avidin/biotin probe could equally well be applied to RIA, or so it seems to me, with similar sensitivities and

times of 2-40 hours depending on how the test is carried out.

The method described on the last line - Circular Intensity Differential Scattering, or CIDS for short - is the technique that I will describe. It is one of the few really new approaches to microbial identification to have come into existence in the past several decades and, while there are many questions yet to be answered, my colleague Dr. Salzman and I feel strongly that this method has the potential of revolutionizing clinical microbiology. It requires times of the order of minutes and, at the moment, something like 5×10^5 organisms per ml. We feel that the sensitivity can be improved by an order of magnitude or more by further modifications of the instrument.

Several generations of instruments are planned. The first of these is a static instrument designed to identify bacteria in pure culture or viruses in typical clinical specimens in a static cuvette system. This machine presently exists in prototype and is that on which most of the data I will present was taken. A second generation machine employs the same principle but with the addition of a flow cytometer so that organisms can be examined one at a time. This machine is intended for bacterial identification. It will be particularly useful in identifying the components in a polymicrobic infection.

The CIDS method involves, unfortunately, concepts that are not the sort that most microbiologists learn at their mother's knee. And the translation of these concepts into a

working instrument involves some complex electronics. As if that weren't enough, the way in which the results are expressed is in an unfamiliar form.

So there are basic communications problems to overcome and these are exacerbated by the position of this paper on the program. There's a wonderful Snoopy cartoon that has Charlie Brown saying, "There's no problem too big for me to run away from." To some extent that's what I propose to do. I will concentrate on the results we have obtained and say comparatively little about the instrumentation or the theory. Fortunately, Dr. Salzman who built the instrument, and who is one of the few people who really understands the theory, is in the audience and he is available to answer questions in these areas.

May I have the next slide, please. This is the definition of CIDS and I'll leave that slide up while I explain a bit further. CIDS may be usefully compared with Circular Dichroism (or CD), which is also defined on this slide. It's important to understand both the similarities and the differences between these two properties. Circular Dichroism is the differential <u>absorption</u> of left and right circularly polarized light and occurs only at an absorption band of the molecule in question, while CIDS is the differential <u>scattering</u> of left and right circularly polarized light, and occurs throughout the spectrum, rather than only at absorption bands.

So we are looking at the differential scattering of polarized light. This is, moreover, not to be confused with

the total light scattering that was investigated so extensively by Wyatt and others. Total light scattering depends only on the size, shape, and average refractive index of microorganisms and did not prove useful for microbial identification.

The next slide is a cartoon to make clear the ideas that I've just presented. It shows light, alternately right and left circularly polarized, impinging on a microbial sample. The sample differentially scatters left and right circularly polarized light with the result that the scattered light is enriched in one component or the other. It is this differential scattering that we are measuring.

What is the physical basis of these measurements in terms of microbial identification? We don't know all the answers to that question yet, but, provisionally we feel that we are measuring the three dimensional "packaging" of helical molecules, and largely that of the microbial genome. Theoretically, the CIDS spectrum is known to depend upon the pitch and radius of the scattering helix. May I have the next slide, please. This shows some of our early work in which the CIDS spectrum of highly purified and supercoiled DNA from plasmid pBr 322 was taken as a function of wavelength and the DNA was then treated with DNAse and the spectrum retaken at intervals. It was finally compared with the CIDS spectrum of commercial calf thymus DNA as a marker for short, linear fragments of DNA. It is clear that the CIDS spectrum changed progressively as the DNA uncoiled.

In the laboratory the CIDS spectrum can be taken as a

function of wavelength, as in the data I just showed; it can also be taken as a function of scattering angle, and as a function of matrix element - a point I'll return to in a moment.

May I have the next slide, please. This shows in block diagram form the geometry of the instrument we are currently using to make measurements as a function of scattering angle. It is this instrument that was used to obtain most of the data we will present here today. I will enumerate briefly the components and what they do.

Light (from a laser or any other convenient source) passes through a polarizer, then through a photoelastic modulator that modulates the polarization at a selected frequency. This alternately left and right circularly polarized light then impinges on a microbial sample in a cuvette and the light is differentially scattered. The detection arm rotates under computer control, stopping at the specified angle and taking data for as long as we tell it too. The detector arm also contains a photoelastic modulator, a polarizer, and finally a photomultiplier tube. What is finally measured is, as described in an earlier slide, the intensities of the scattered left and right circularly polarized light. The spectrometer that we use is a variant of one designed by Thompson et al. at the University of Texas[4]; other pioneering work in this area has been done by Hunt and Huffman and by Bickel and his colleagues at the University of Arizona[5-7] and especially by Bustamante, Tinoco, Maestre, and their coworkers at the University of Cal-

ifornia, Berkeley.[8-14] Some of our own work has been published as well.[15-16] Additional theoretical work has been published by Zietz et al.[17]

There is one additional complexity that must be discussed before we turn to the data. To put it as simply as possible, the polarization properties of the light beam at each point in the instrument is described by a Mueller matrix. May I have the next slide, please. The Mueller matrix consists of sixteen elements described as S_{11}, S_{12}, etc. Technically, only the S_{14} element of the Mueller matrix is the CIDS parameter, so we are being imprecise in describing the results of using other matrix elements as CIDS. Unfortunately, we don't have a better name for the technology at the moment.

Each of these matrix elements can be measured by measuring different frequencies in the output signal. In the instrument that Dr. Salzman is now building as a prototype for a commercial instrument it will be possible to measure eight of the sixteen Mueller matrix elements, each of which brings some additional information to the problem of microbial identification. This is an extraordinarily powerful tool that we have only begun to explore the uses of.

To summarize, then, "CIDS" spectra - with CIDS in quotation marks, can be measured as a function of wavelength, scattering angle, and/or matrix element, and a number of matrix elements can be measured (virtually) simultaneously. This panoply of measurements potentially gives the method resolving power for microbial identification that we have

only just begun to explore.

I would now like to present some representative data that we have taken over the past couple of years. Because of the limitations of the laboratory we now occupy we were unable to use live pathogens, so much of the data I will present is on vaccine preparations or other kinds of inactivated material. This is obviously a source of artifacts. In addition, we now have a better machine and better ways of expressing the data, and we will shortly begin to repeat some of these experiments in the facilities that we plan to move into in August. So the data I will present must be regarded as preliminary and are offered as "proof of principle" experiments rather than as definitive.

May I have the next slide, please. This slide shows CIDS spectra as a function of scattering angle for three type A influenza virus vaccine preparations in egg allantoic fluid given to us by our colleagues in the Center for Infectious Diseases of the Centers for Disease Control, Atlanta. Although these are certainly not pure virus preparations the spectra of the three viral strains are clearly separable. At the time this work was done it took about 20 minutes to run each of these spectra, and the presence of egg allanotic fluid did not prevent the three type A viruses from giving distinct CIDS spectra.

May I have the next slide, please. Our colleagues at CDC wondered if perhaps the differences in the CIDS spectra were due to differences in GC content, as in the flow cytometric method of Van Dilla and his colleagues[12]. So they

sent us samples of three very different bacteria that have essentially the same GC content. These are pure bacterial cultures run separately, with the CIDS spectra presented as a function of wavelength. As you can see there are angular regions, particularly between 130 and 140 degrees in which the separation among the spectra is good, It is also highly reproducible.

May I have the next slide, please. This shows the CIDS spectra as a function of wavelength for the four types of dengue fever virus as vaccine preparations, also supplied by CDC. Particularly in the region around 30^{o}, the four viral types are readily distinguishable within the experimental variation indicated by the error bars. Note that all the dengue fever preparations have large negative signals in this region of the spectrum.

May I have the next slide, please. These are similar data for four types of encephalitis virus vaccines from CDC. Again, the region of maximum resolution is about 30^{o}. As you know it is difficult to distinguish St. Louis Encephalitis Virus from Dengue Fever virus serologically. The CIDS spectra of the vaccine preparations are, however, very different.

May I have the next slide, please. We have also done some recent experiments with crude hepatitis B viral preparations kindly given us by Warner-Lambert Corporation, and compared their spectra with those of Dengue Fever virus vaccine and Eastern Encephalitis vaccine. Here we have employed the additional discrimination provided by measuring

two matrix elements, S_{14}, the true CIDS element, and S_{34}. When both matrix elements are employed there is good separation among these three types of preparation. The use of additional matrix elements should give increasingly enhanced resolution and, I remind you, we have eight matrix elements that we can measure simultaneously.

May I have the next slide, please. This slide represents an effort to determine the sensitivity of the CIDS technique for determining hepatitis virus in clinical material. According to theory the CIDS signal - you'll recall that it's a ratio of the sums and differences of intensities - should be independent of concentration. This slide shows a plot of the CIDS signal as a function of hepatitis DNA concentration. We can get usable CIDS spectra down to levels of 1 ng of hepatitis B viral DNA/ml with no real effort made to maximize sensitivity.

This sort of data always bothers people, however, because they expect that a proper dilution curve should go through zero. As shown here, with CIDS a dilution curve is a straight line parallel to the x axis until the point at which the signal to noise ratio is so unfavorable that the signal can no longer be distinguished. This is far from a system optimized for maximal sensitivity. Dr. Salzman feels that we can improve the sensitivity by at least an order of magnitude by some relatively simple modifications of the instrument.

To summarize: We feel that this technique has immense potential although there are many obvious questions still be

to be answered. You'll be hearing from us at intervals as we extend our understanding of the strengths and limitations of this technique for rapid identification of microorganisms in clinical material.

Acknowledgements

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References

1. Isenberg H (1982) Clinical significance of automation in microbiology. In: Tilton, RC (ed) Rapid methods and automation in microbiology. American society for microbiology, Washington, D.C. pp 96-97

2. Tilton RC, Balows A (1983) Introduction: Body fluids and infectious disease - clinical and microbiological advances. Am J Med 75 1

3. Jenista JA, Prather, SP, Powell, KR, Menegus, MA (1983) Virus cultures in the reduction of neonatal morbidity. Ped Rec 17 224A (Abstr. #824)

4. Thompson RC, Bottiger JR, Fry ES (1980) Measurement of polarized light interactions via the Mueller matrix. Appl Opt 19 1323-1332

5. Hunt AJ, Huffman, DR (1973) A new polarization-modulated light scattering instrument. Rev Sci Instrum 44 1753-1762

6. Bickel WS, Davidson JF, Huffman DR, Kilkson R (1976) Application of polarization effects in light scattering: a new biophysical tool. Proc Nat Acad Sci USA 73 496-490

7. Bickel WS, Stafford ME (1981) Polarized light scattering from biological systems: a technique for cell differentiation. J Biol Phys 9 53-66

8. Maestre MF, Gray DM, Cook RB (1971) Magnetic circular dichroism study in synthetic polynucloetides, bacteriophage structures and DNAs. Biopolymers 10 2537-2553

9. Bustamante C, Maestre MF, Tinoco I Jr (1980) Circular intensity differential scattering of light by helical structures I. Theory. J Chem Phys 73 4273-4281

10. Bustamante C, Maestre MF, Tinoco I Jr (1980) Circular intensity differential scattering of light by helical structures II. Applications. J Chem Phys 73 6046-6055

11. Bustamante C, Maestre MF, Tinoco I Jr (1981) Circular intensity differential scattering of light by helical structures III. A general polarizability tensor and anomalous scattering. J. Chem Phys 74 4839-4850

12. Maestre, MF, Katz J (1982) A circular dicrhosim microspectrophotometer. Biopolymers 21 1899-1908

13. Tinoco I Jr, Bustamante C, Maestre MF (1982) Chiroptical methods and their applications to biomolecular structures. In: NATO adv study inst Ser A 45 269-303

14. Van Dilla MA, Langlois RG, Pinkel D, Yajko D, Hedley, WK (1983) Bacterial characterization by flow cytometry. Science 220 620-622

15. Salzman GC, Griffith JK, Gregg CT (1982) Rapid identification of microorganisms by circular intensity differential scattering. Appl and Environ Microbiol 44 1081-1085

16. Salzman GC, Gregg CT (1984) Current and experimental methods of rapid microbial identification. Bio/Technology March

17. Zeit- S Belmont A, Nicolini C (1983) Differential scattering of circularly polarized light as a unique probe of polynucleosome superstructures. A simulation by multiple scattering of dipoles. Cell Biophysics 5 163-187

RAPID METHODS OF MICROBIAL IDENTIFICATION

	REQUIREMENTS	
TEST	TIME-HOURS	ORGANISMS/ML
COAGGLUTINATION	1	10^6-10^7
COUNTERIMMUNOELECTROPHORESIS	1	10^6-10^7
FLUORESCENT ANTIBODY STAINING	1	$>10^5$
ELISA (OR ELISA A/B)	4-6	10^3-10^4
RADIOIMMUNOASSAY	2-40	10^3-10^4
CIRCULAR INTENSITY DIFFERENTIAL SCATTERING (CIDS)	<0.2	$<5 \times 10^5$

Figure captions:

Fig. 1. Definition of Circular Dichroism (CD) and Circular Intensity Differential Scattering

Fig. 2. Cartoon illustrating the CIDS principle

Fig. 3. Effect on the CIDS spectra as a function of wavelength of DNAse treatment of highly supercoiled plasmid DNA. The spectrum of calf thymus DNA (linear, small fragments) is shown for comparison

Fig. 4 Block diagram of the angular-scanning CIDS spectrometer

Fig. 5 CIDS spectra as a function of angle at 488nm of three type A influenza virus vaccine preparations. Good discrimination is obtained among these three virus preparations around 60^{o}, 110^{o}, and 150^{o}

Fig. 6 The CIDS spectra at 488nm as a function of angle for three bacteria of very nearly the same GC content. The spectra were run on separate suspensions of each bacterium in pure culture. Resolution among the four spectra is best at $120\text{-}140^{o}$

Fig. 7 The CIDS spectra at 360nm of four types of dengue fever virus vaccines. The best resolution among the four viral vaccine types is obtained near 30^{o}

Fig. 8 The CIDS spectra at 360nm of four types of encephalitis virus vaccines. Best resolution among the four viral vaccine types is obtained near 30^{o}

Fig. 9 The resolution obtained at 488nm and 112^{o} of a crude formalin-treated hepatitis B virus preparation (Dane

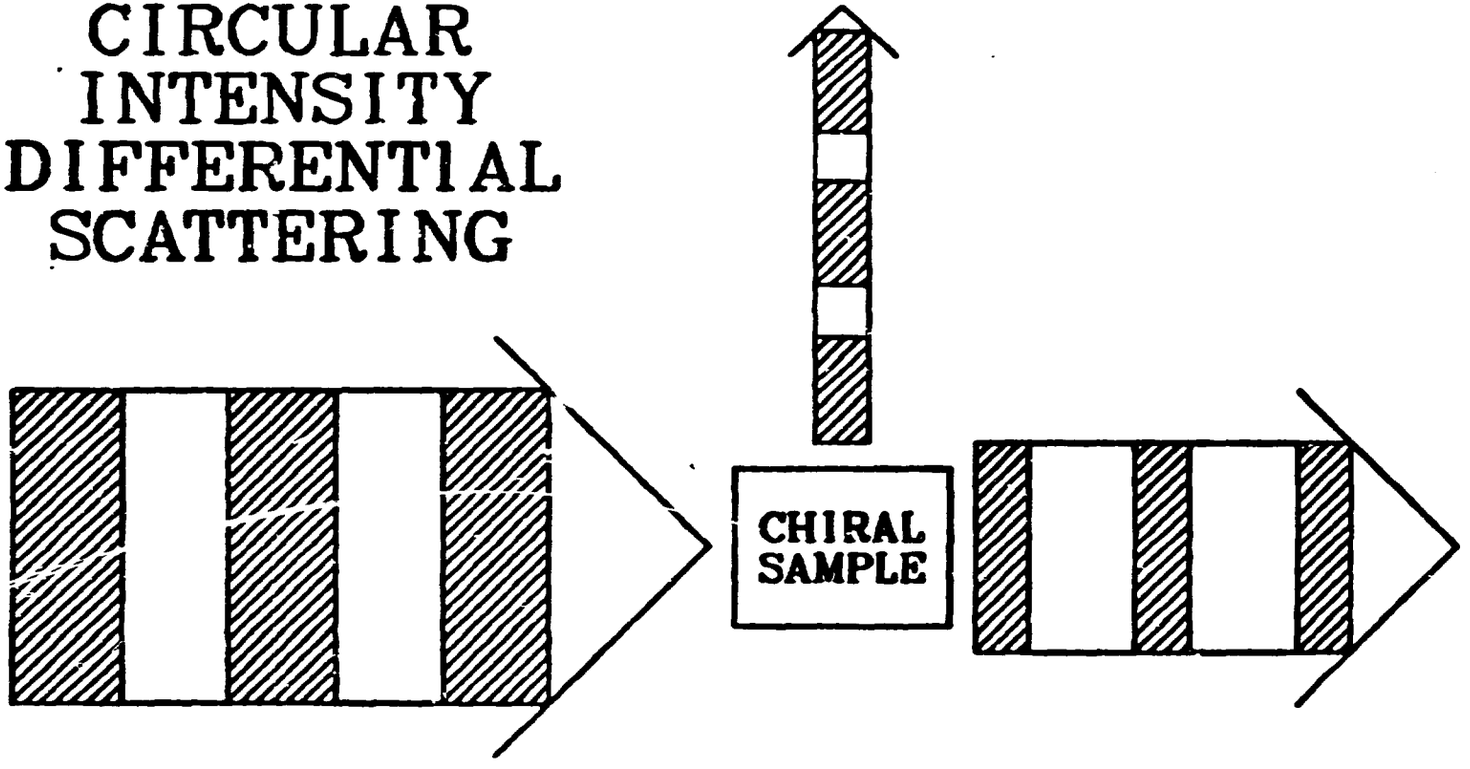
particles, surface antigen, etc), a dengue fever vaccine preparation, and a Venezuelan Equine Encephalitis vaccine prep aration when the "CIDS" signal from two Mueller matrix elements, $F_{1,4}$ (true CIDS) and $F_{3,4}$, are employed

Fig. 10 The detection limit of the present CIDS spectrometer for the detection of crude formalin-fixed hepatitis B virus preparation at 488nm and 112^{o}. One ng of viral preparation DNA per ml

RAPID METHODS OF MICROBIAL IDENTIFICATION

TEST	REQUIREMENTS TIME-HOURS	ORGANISMS/ML
COAGGLUTINATION	1	10^6-10^7
COUNTERIMMUNOELECTROPHORESIS	1	10^6-10^7
FLUORESCENT ANTIBODY STAINING	1	$>10^5$
ELISA (OR ELISA A/B)	4-6	10^3-10^4
RADIOIMMUNOASSAY	2-40	10^3-10^4
CIRCULAR INTENSITY DIFFERENTIAL SCATTERING (CIDS)	<0.2	$<5 \times 10^5$

CIRCULAR INTENSITY DIFFERENTIAL SCATTERING



$$CIDS(\theta) = (I_L(\theta) - I_R(\theta)) / (I_L(\theta) + I_R(\theta))$$

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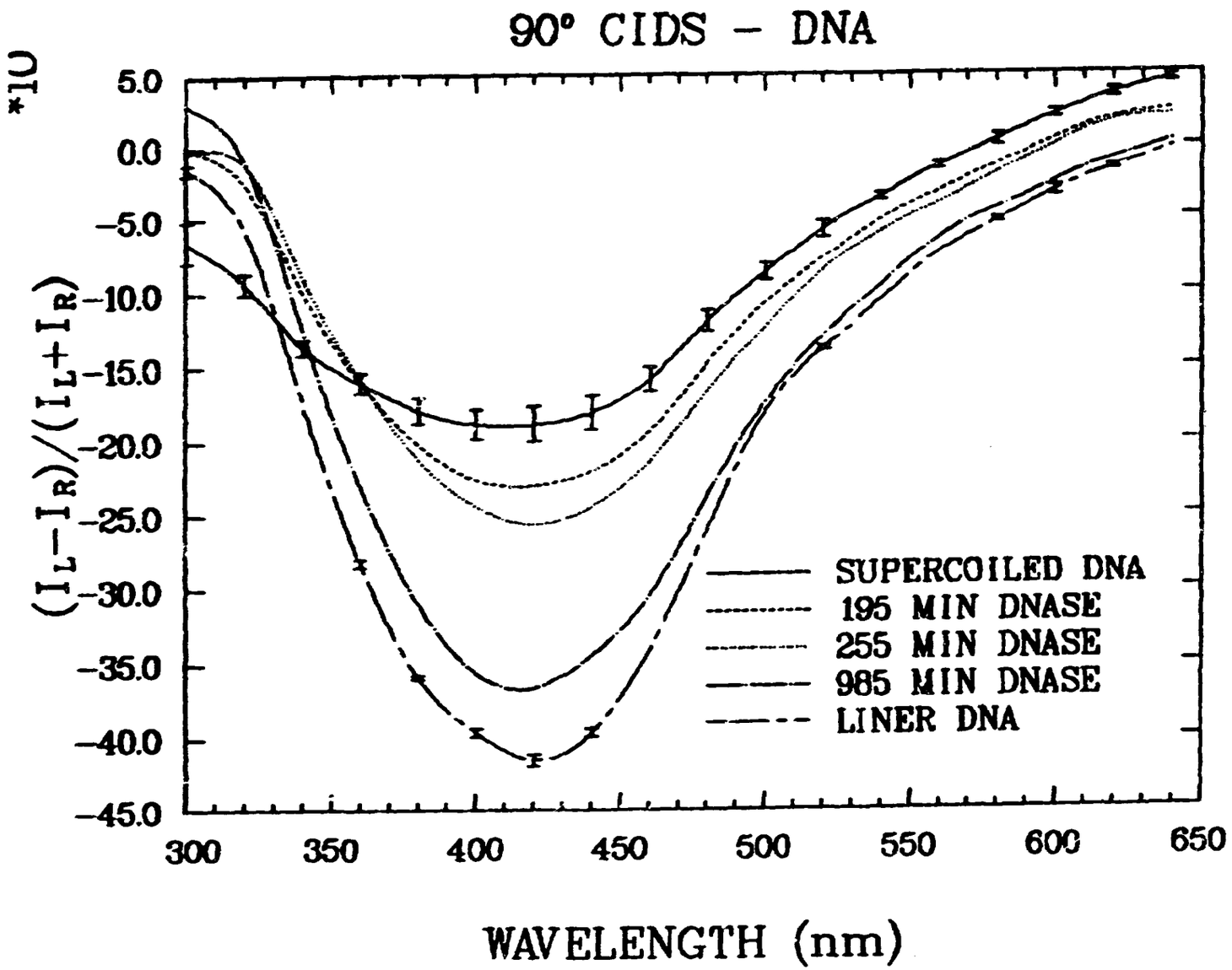
Fig 1

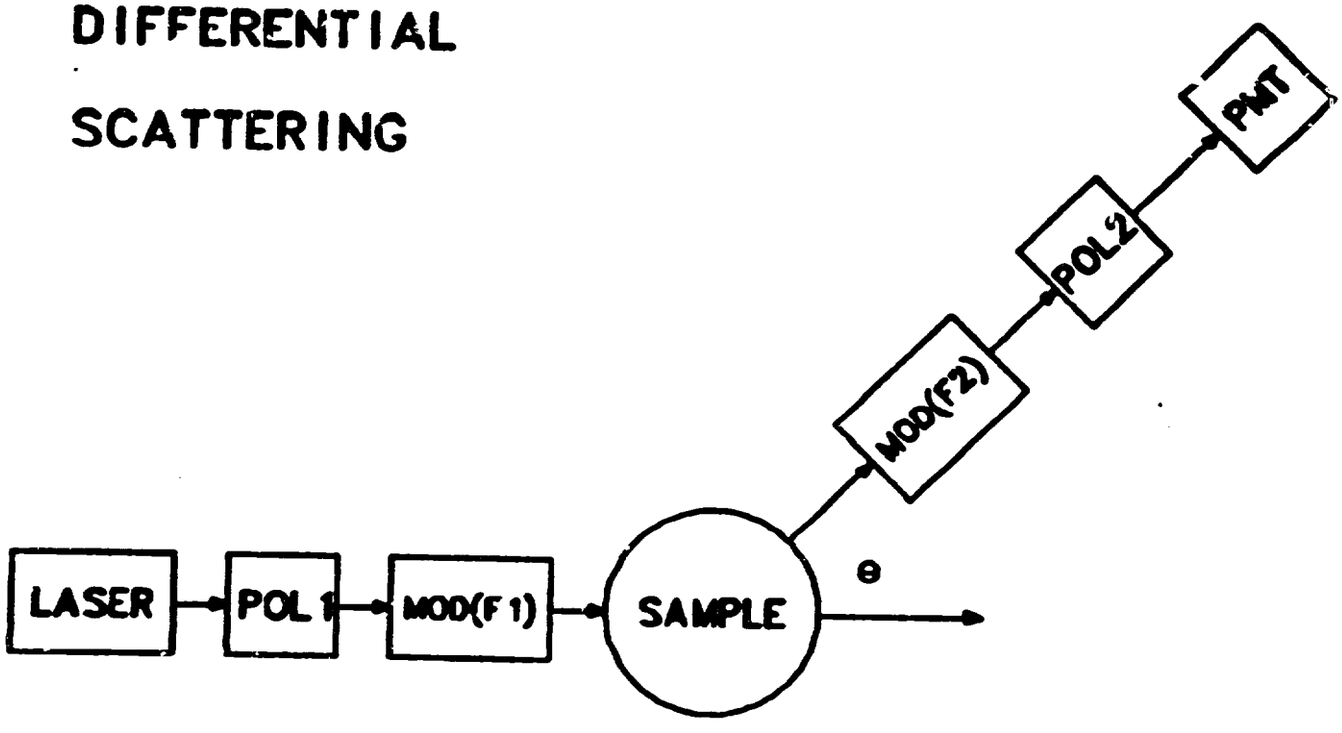

90° CIDS – DNA
*10
5.0
0.0
-5.0
-10.0
-15.0
-20.0
-25.0
-30.0
-35.0
-40.0
-45.0
$(I_L-I_R)/(I_L+I_R)$
300
350
400
450
500
550
600
650
WAVELENGTH (nm)
SUPERCOILED DNA
195 MIN DNASE
255 MIN DNASE
985 MIN DNASE
LINER DNA

CIRCULAR

INTENSITY

DIFFERENTIAL

SCATTERING



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MUELLER MATRIX

F_{11}(0)	F_{12}(–)	F_{13}(100)	F_{14}(50)
F_{21}(–)	F_{22}(–)	F_{23}(–)	F_{24}(–)
F_{31}(94)	F_{32}(–)	F_{33}(6)	F_{34}(44)
F_{41}(47)	F_{42}(–)	F_{43}(53)	F_{44}(97)

ϵ_1=45 γ_1=90 50kHz

θ_2=45 γ_2=90 47kHz

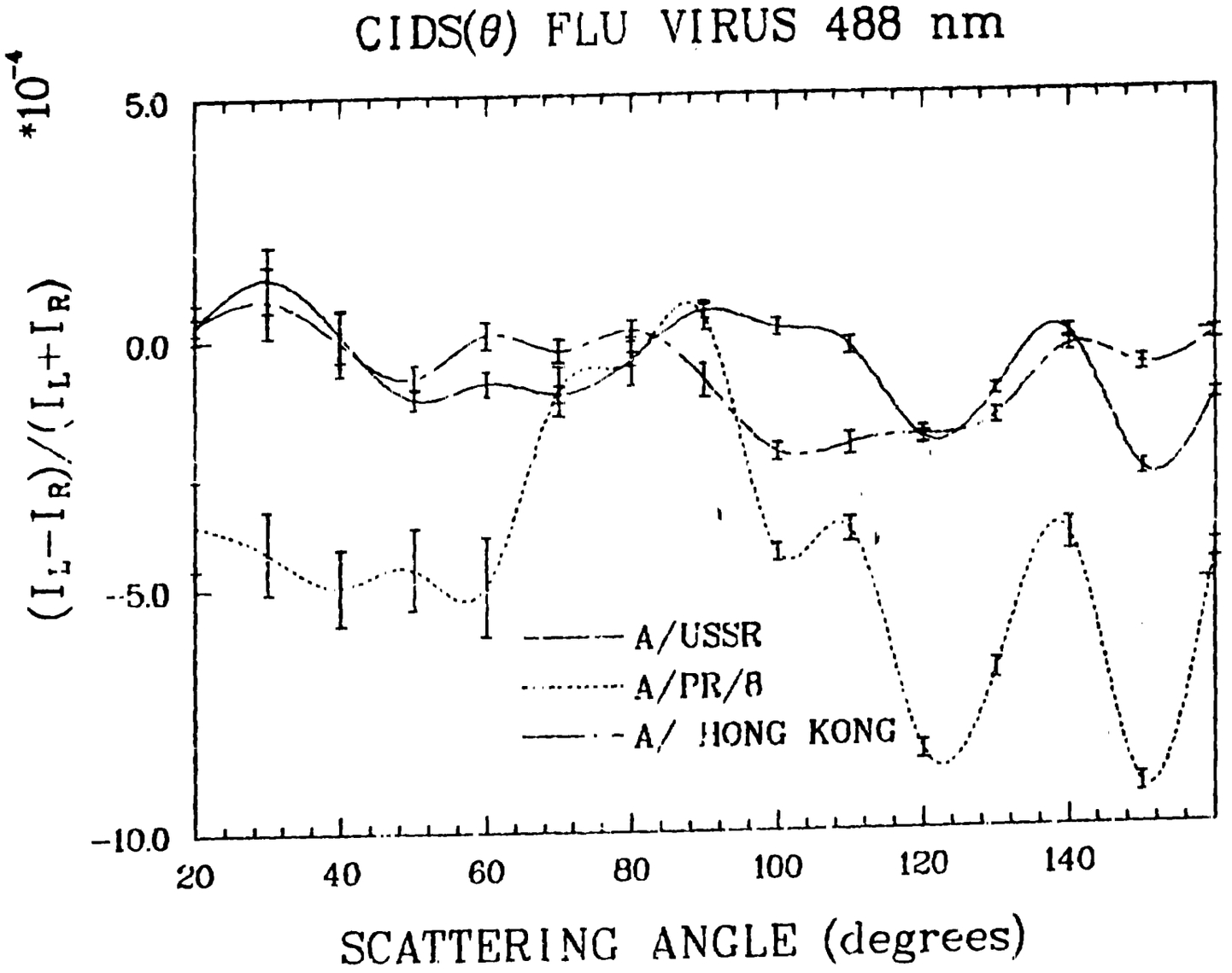
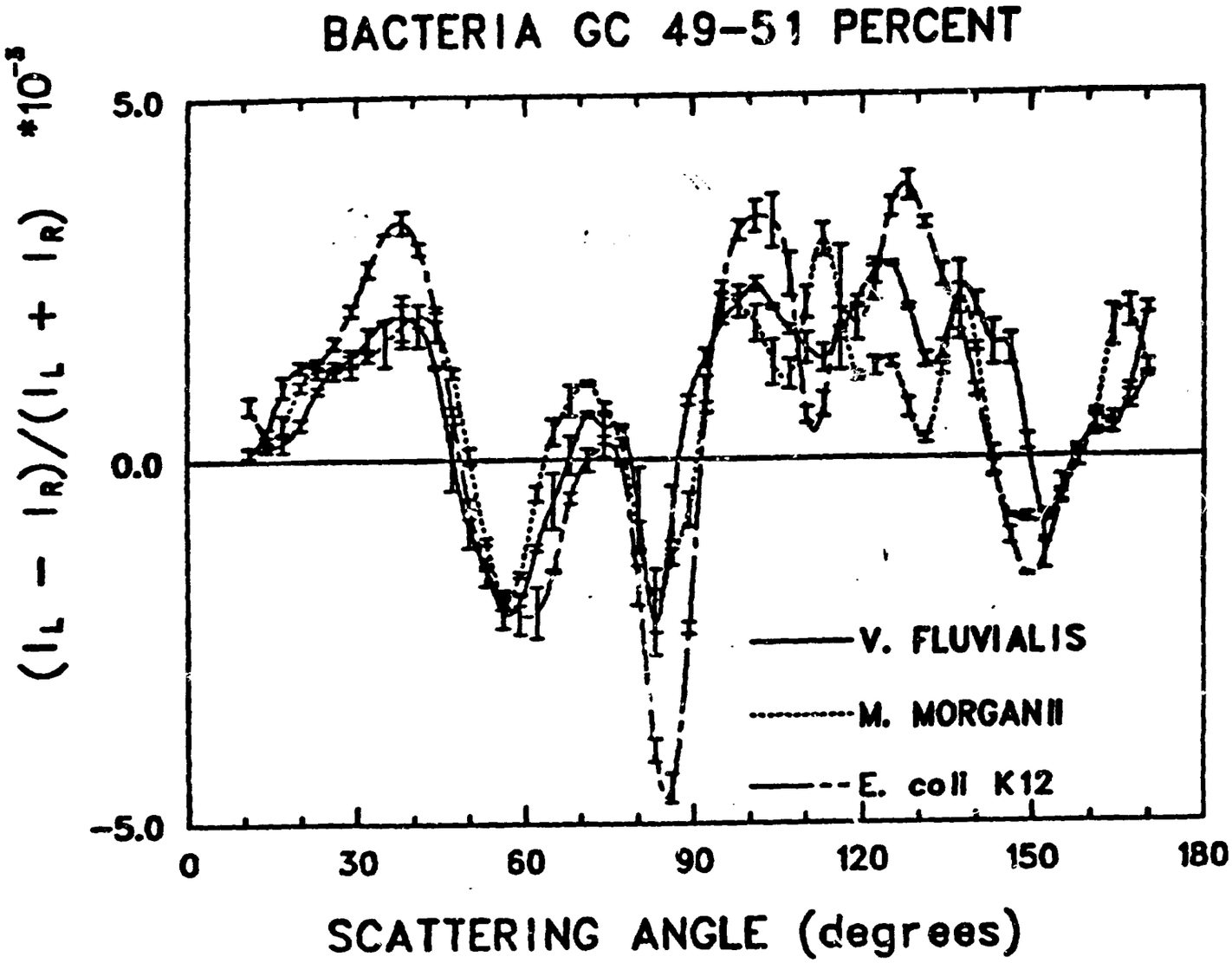
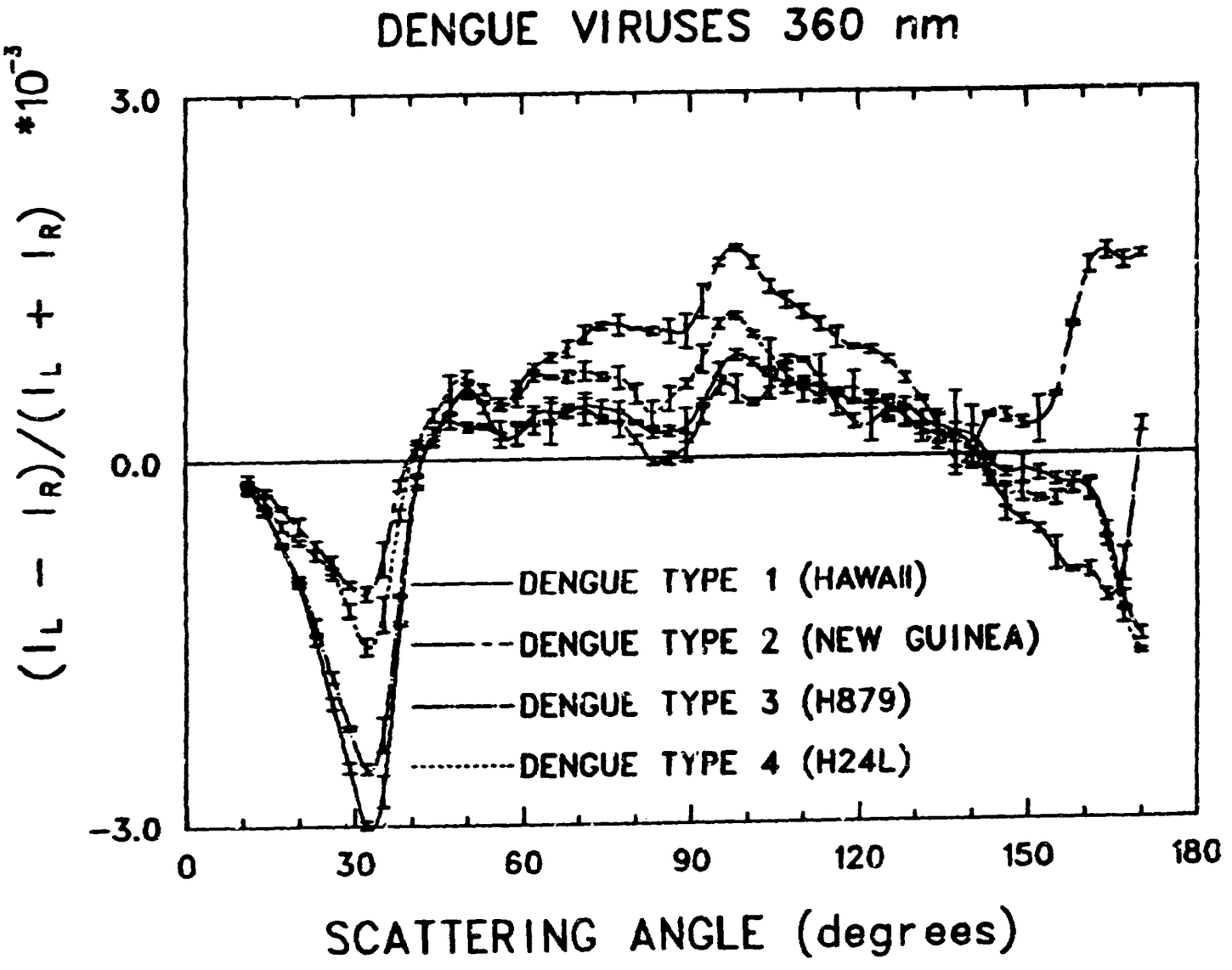
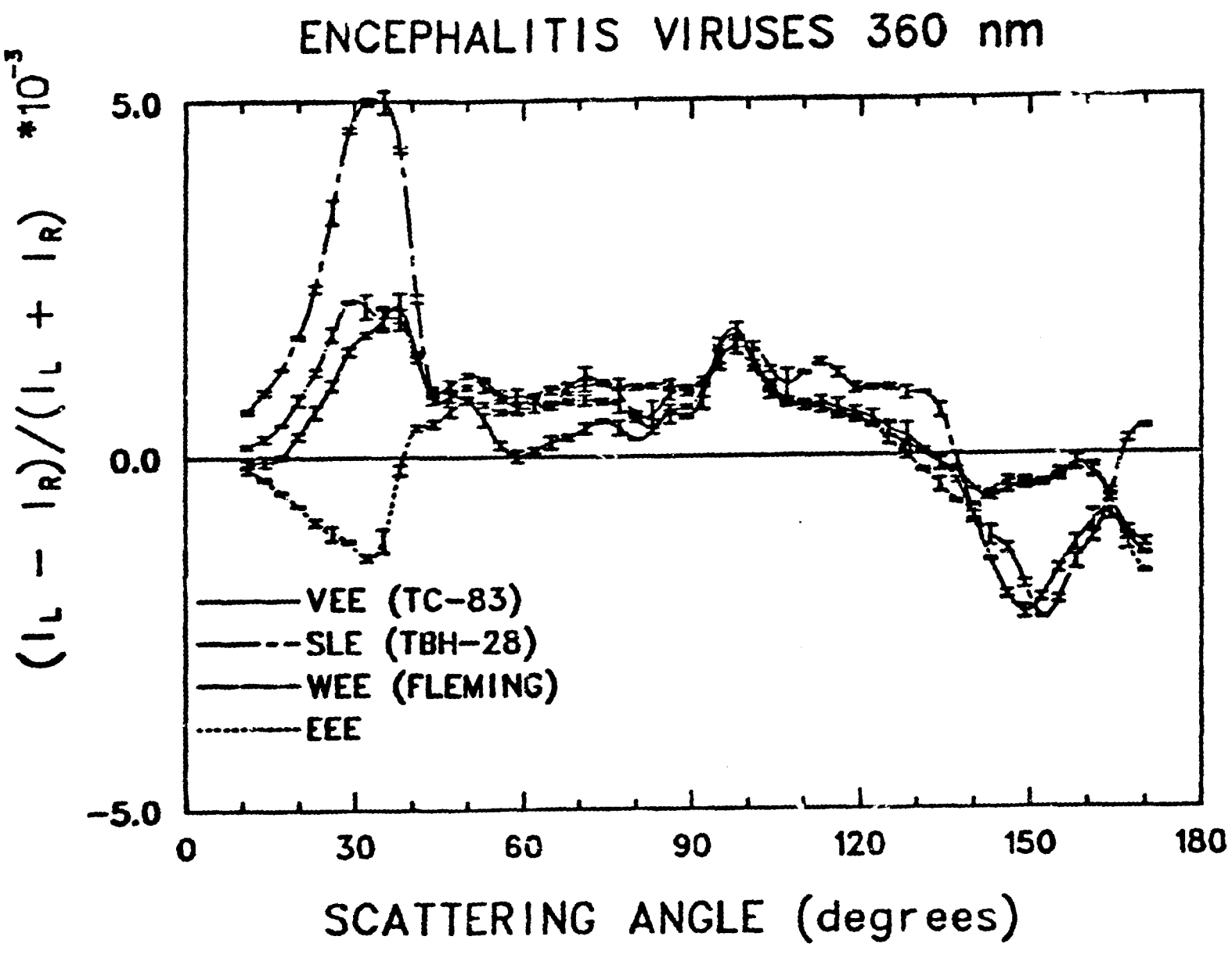

CIDS(θ) FLU VIRUS 488 nm
*10⁻⁴
(I_L−I_R)/(I_L+I_R)
5.0
0.0
-5.0
-10.0
20
40
60
80
100
120
140
SCATTERING ANGLE (degrees)
A/USSR
A/PR/8
A/ HONG KONG

Fig 3



MORGIMP
Fig 4


DENGUE VIRUSES 360 nm
$(I_L - I_R)/(I_L + I_R)$ $*10^{-3}$
3.0
0.0
-3.0
0
30
60
90
120
150
180
SCATTERING ANGLE (degrees)
DENGUE TYPE 1 (HAWAII)
DENGUE TYPE 2 (NEW GUINEA)
DENGUE TYPE 3 (H879)
DENGUE TYPE 4 (H24L)


ENCEPHALITIS VIRUSES 360 nm
$(I_R)/(I_L + I_R)$ *10^{-3}
5.0
0.0
-5.0
VEE (TC-83)
SLE (TBH-28)
WEE (FLEMING)
EEE
0
30
60
90
120
150
180
SCATTERING ANGLE (degrees)

Resolving Power

~~Accuracy~~ in $S_{1,4}$ and $S_{3,4}$.