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Serologic Test Development

July 1, 1974—June 30, 1975

by

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SEROLOGIC TEST DEVELOPMENT ANNUAL REPORT

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ABSTRACT

The Enzyme-Labeled Antibody (ELA) test system has been adapted to microtiter trays for both cell bound and soluble antigens. Problems involving both readout instrumentation and reaction product stability have been solved. Progress involving application of the ELA system for detection of hog cholera, trichinosis, swine brucellosis, and swine and bovine tuberculosis is reported. Prototype instrumentation for automating ELA processing is being developed.

I. INTRODUCTION

Through an interagency agreement between the United States Department of Agriculture (USDA) and the United States Energy Research and Development Administration (ERDA), the Los Alamos Scientific Laboratory (LASL) has approached disease surveillance problems by application of biological and physical sciences. One major goal of the USDA/ERDA interaction has been to develop a rapid, singular test system with adequate sensitivity and specificity for the detection of antibodies to a multiplicity of diseases. The test, once automated, must be able to keep up with slaughter plant processing times, but the system should also possess characteristics such that a simple and reliable field test can be developed. Preliminary data suggest that, with some refinements, the Enzyme-Labeled Antibody (ELA) methodology developed at LASL appears to meet the above boundary conditions. Confidence exists that reagents can be prepared and test limits can be set to establish the sensitivity and specificity required for a national disease surveillance program.

This report is organized into (1) a section which describes test refinements accomplished to both simplify automation design and to increase the sensitivity of the test, (2) several sections which describe work done on the various diseases to which we are attempting to adapt the test, and (3) a section which describes progress in instrumentation development.

II. ENZYME-LABELED ANTIBODY (ELA) TEST REFINEMENTS

The use of microtiter trays as the antigen carrier for the ELA test has aided in the progress of adapting the manual processing procedures to an automated one; automation of ELA is one of our more important long-range goals. The accuracy of the test has been improved through development of both a simple readout instrument and the stabilization of reaction-product solutions.

A. Adaption of Microtiter Trays

A significant procedural advance in the ELA test occurred when 96-well disposable microtiter trays proved to be suitable antigen and test vehicles. Methodology concerning this advance has been detailed in a paper submitted to the Journal of Clinical Microbiology entitled, "A Simplified Micromethod of Screening for Antibodies to Disease Agents Using the Indirect Enzyme-Labeled Antibody (ELA) Concept." The well trays will greatly simplify the

development of instrumentation designed to automate test processing.

B. Readout Developments

The design and construction of a self-contained colorimetric readout system has been most helpful to our experiments. There is very little drift in this instrument, and consequently, large numbers of samples can be read in a single sitting without having to continually recalibrate the instrument. A detailed description of this instrument is found in Section VIII.

C. Studies on Substrate and Product

To better evaluate the amount and purity of antigens and antibodies being used, it is best to quantitate the ELA assay. The absorption properties of the substrate and the reaction product were measured under various conditions to find the optimum condition for the test. One important parameter is the choice of wavelength in the absorption measurements. This was determined by placing the H_2O_2 and aminosalicylic acid substrates in the reference cell of the spectrophotometer and the reaction product in the sample cell. This arrangement gives the "difference" spectrum. The difference spectra for various solutions are shown in Fig. 1. The reason for these measurements is that product and substrate are both present when the absorption measurements are made on samples. If the absorption of the product in the sample was measured at wavelengths where the substrate also absorbed, e.g., 300 nm, then the measurement of product would be confused by the decrease in substrate absorption as it was being converted to product.

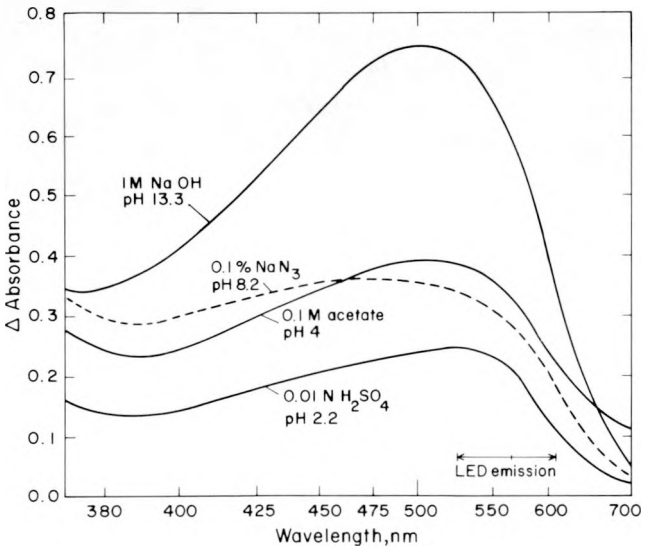

Fig. 1. Difference spectra of enzyme reaction product in various solutions.

The concentration of product is the same in all four spectra, showing that the absorption of the product is dependent on pH. From the spectra it can be seen that 500 nm is about the best wavelength at which to measure the product. The emission of the light emitting diode (LED) used in the colorimetric readout is shown below and is close to the maximum absorption of the product. The absorption of the product in 1 M NaOH, pH 13.3, is about twice that in 0.1 M acetate pH 4. Therefore, the assay would be about twice as sensitive if 1 M NaOH were used. However, there is a complication; namely, that the substrates, 5-aminosalicylic acid and H_2O_2 are unstable at basic pH's, so if 1 M MaOH were used to dilute the assay mixture, any remaining substrate could be converted to product and would subsequently give erroneous readings. Therefore, if this is used it must be added immediately before reading. The substrates are much more stable in 0.1 M acetate pH 4. However, at pH 2.2 in H_2SO_4, the product forms a precipitate and this causes fluctuations in the readings. In the routine testing, 0.005 N H_2SO_4 is used to stabilize the reaction product without causing appreciable precipitation.

We also addressed the question, "Under the conditions of the assay, at what point does the curve of product versus time after mixing substrate and enzyme deviate from linearity?" When this happens, the product is no longer a quantitative estimation of the amount of antibody found. Figure 2 shows the product increase as a function of time after mixing with the enzyme. The times given on the abscissa are when the enzyme activity was stopped by mixing it with NaN_3. The ordinate is the ELA reading (blank minus sample recorded in millivolts) and the absorbance at 500 nm (sample minus blank). The initial concentration of substrate was 0.1 ml of the solution normally used in ELA. Enzyme activity was stopped and then 1 ml 0.1 M acetate was added. It can be seen that under these conditions, the maximum reading on the linear curve, using the ELA readout is about 325, and using absorbance at 500 nm is about 0.24. The deviation from linearity is due to

reduced amounts of substrate available for reaction and possibly product inhibition.

To determine how stable the product and substrate are in 0.1 M acetate pH 4, the last 4 solutions were reread after 16 hours. As shown by the circles in Fig. 2, the readings are somewhat lower. This is because the readings are always a difference reading between the product at a certain time after enzyme addition and the reading of the blank. After 16 hours, some of the substrate in the blank had been converted to product; and thus, the difference readings changed appreciably.

III. HOG CHOLERA

Hog cholera has been the prototype virus disease for detection by ELA. The solving of the numerous problems we have encountered in hog cholera test development will make application of ELA to other important virus diseases, (e.g., African swine fever) a much easier task when the need arises.

A. Soluble Antigen Development

Various procedures were tried to produce large amounts of Hog Cholera Virus (HCV) antigens and to stabilize them. To produce large amounts of PK-15* cells, various conditions for growing PK-15 cells in spinner flasks were tried. The method that appeared to have some promise was growing cells in T-75 culture flasks** with F-10 media instead of F-15, and then transferring these cells to a spinner flask using F-10 without added calcium. The cells went from a concentration of $7 \cdot 10^4$ to $44 \cdot 10^4$ cells/ml. However, the doubling time was very long (72 hours compared to about 24 hours on plastic) and indicates that optimum conditions were not reached. This was not pursued because HCV was considered to be eradicated and efforts here on HCV were redirected. The work was directed to obtaining an evaluation of the ELA test on hog cholera virus infected cells instead of on soluble antigen, and to a greater effort into developing ELA for swine tuberculosis and brucellosis.

Another study involved the effect of pH on the growth of PK-15 cells. For this study, the $NaHCO_3$ in the F-15 media was replaced by the organic buffers N-2-hydrozyethylpiperazine N'-2 ethanesulfonic acid (HEPES) and piperazine N,N' bis 2-ethanesulfonic acid (PIPES) and the ionic strength was adjusted to that of F-15 media with NaCl. With these changes,

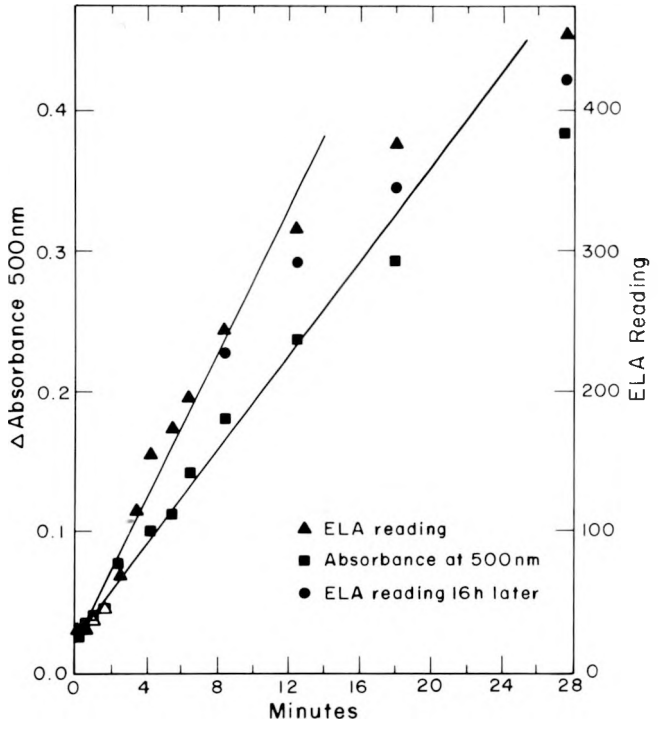

Fig. 2. Measurement of product formed versus time, after mixing substrate and enzyme using the ELA readout and a Beckman spectrophotometer.

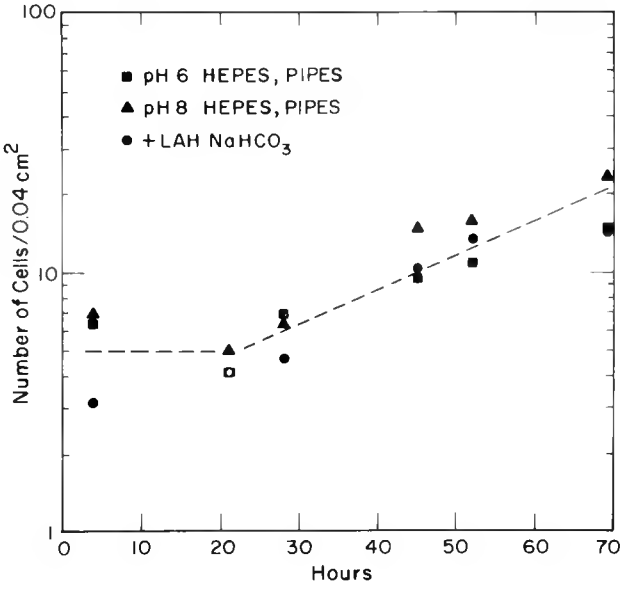

Fig. 3. PK-15 cell growth under various conditions.

*Obtained from Veterinary Services Laboratories in Ames, IA.

**Obtained from Grand Island Biological Company, Grand Island, NY.

the pH could easily be altered and not be dependent on a CO_2 atmosphere. Figure 3 shows the cell growth curve for HEPES, PIPES, pH 6 and pH 8 without lactalbumin hydrolyzate (LAH), compared to the conventional way of growing PK-15 cells in F-15 with LAH and $NaCO_3$ buffer. Initially, the cells appear to grow at the same rate with a doubling time of approximately 25 hours. Table I shows the final numbers of cells reached in each 19.7 cm^2 dish. The highest number reached is by the conventional procedure, but the HEPES, PIPES pH 7.5 media with $47.7 \cdot 10^4$ cells is very close to the $NaHCO_3$ buffered media with $67.4 \cdot 10^4$ cells, so it appears that the HEPES, PIPES media supports good growth of PK-15 cells. It also appears that LAH, although not essential for growth, produces more cells. The main reason these experiments were started was to see the change in antigen production at these various pH's, but again, this was not pursued due to redirection of efforts.

Elution and solubilization of the antigenic material from the PK-15 HCV material was tried using various reagents. The infected cells were collected and freeze-thawed in PBS three times and centrifuged. The supernate was tested by ELA and the precipitate was extracted with guanidine hydrochloride or with Triton X-100. The following signal-to-noise ratios (S/N) were obtained in the ELA test:

Sample	S/N
Phosphate buffered saline (PBS) Supernate	1.3
13% Triton X-100	2.2
7 M Guanidine	1.1

Thus, it appears that Triton X-100 can solubilize some of the antigen. Finally, various reagents were tested to see if they could increase antigen stability. Neither various pH's nor mercaptoethanol were found to be successful.

B. Hog Cholera Serum Bank

The Veterinary Services Laboratories (VSL) hog cholera serum bank (640 samples) was tested blind using infected cells grown on microtiter trays. The results were disappointing in that there were 36 false positives and 34 false negatives. Some blocks were entirely correct, while others had numerous errors. However, part of the purpose of screening the serum bank was to define and correct problem areas. Several causes for these erratic results have been identified. First, the serum bank was done before we had the new readout instrument. There was considerable drift in the old readout; this drift probably caused some overlap in either direction between positive and negative values. Second, under the conditions utilized at the time, the reaction product was not stable. We later discovered that when reaction product is transferred into distilled water for reading, there is a rapid decrease in the readout value over a period of 8 to 10 minutes; this has been corrected by transferring the reaction product into 0.005 N H_2SO_4. The lower pH stabilizes the reaction product (see Sec. I.C).

A third important finding has been the discovery that the fixation conditions of the hog cholera-infected cells were not optimum. We used a mixture of 35% acetone and 65% saline to fix the antigen used in the serum bank trial. Table II illustrates that concentrations of acetone above 40% totally destroy the antigen activity of the infected cells.

TABLE I

GROWTH OF PK-15 CELLS AT VARIOUS pH's

Conditions	Total # Cells
pH 6.0 - LAH	$3.6 \cdot 10^4$
pH 6.5 - LAH	$24.9 \cdot 10^4$
pH 7.0 - LAH	$42.6 \cdot 10^4$
pH 7.5 - LAH	$47.7 \cdot 10^4$
pH 8.0 - LAH	$11.6 \cdot 10^4$
$NaHCO_3$ + LAH	$110.7 \cdot 10^4$
$NaHCO_3$ - LAH	$67.4 \cdot 10^4$

TABLE II

EFFECT OF ACETONE CONCENTRATION ON THE ANTIGENIC ACTIVITY OF HOG CHOLERA INFECTED PK-15 CELLS GROWN ON PLASTIC[a]

Acetone Concentration (%)[b]	ELA UNITS[c] Hog Cholera Negative Serum	Hog Cholera Positive Serum	S/N Ratio[d]
20	17	58	3.4
30	13	35	2.7
40	11	13	1.2
70	6	5	0.8
80	31	37	1.2

[a] Fixed for five minutes at room temperature.

[b] In 0.15 M saline.

[c] Mean of three replicates.

[d] Obtained by dividing the net positive reading by the net negative reading.

TABLE III

DOUBLE BLIND TEST OF SWINE SERA FOR ANTIBODIES TO T. spiralis

RESULTS OF SAFA AND ELA TESTS

Pig No.	Larvae Fed	PI* S**	PI* E†	Day 7 S	Day 7 E	Day 14 S	Day 14 E	Day 17 S	Day 17 E	Day 21 S	Day 21 E	Day 28 S	Day 28 E	Day 35 S	Day 35 E	Day 42 S	Day 42 E	Day 168 S	Day 168 E
6470	0			-	-														
6674	0	-	-	-	-														
6473	0	-	-	-	-														
6502	0	-	+	-	+														
6571	0	-	-													+	+		
6491	25	-	-	-	+	+	+			+	+	+	+			+	+	+	+
6500	25	-	-			-	-			-	-	+	+			+	+	+	+
1007	100	-	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+		
0204	500	-	-	+	+	-	+	-	-	+	+			+	+	+	+		
0250	500			-	+	-	+	-	-	+	+	+	+	+	+	+	+		
6988	500	-	-	-	-	+	-	-	+	+	+	+	+			+	+		
6501	2,500	-	-	-	-	+	+			+	+					+	+	+	+
6572	2,500	-	-	-	+	-	+			+	+	+	+					+	+
6504	250,000	-	-	-	+	+	+			+	+	+	+			+	+	+	+
6483	250,000	-	-	-	-	+	+			+	+	+	+			+	+	+	+
6570	250,000	-	-	+	+	+	+			+	+	+	+			+	+	+	+

*PI = Pre-immune sera **S = SAFA test result †E = ELA test result

This may be due to the interaction between the solvent, the plastic, and the cells. In any case, 20% acetone appears to satisfactorily fix the cells and preserve antigenic activity. We believe the nonoptimal fixation of the antigen was responsible for a significant number of the false negative reactions. Finally, we believe a large number of the false positive reactions were caused by using the wrong type of negative standard; that is, a serum pool obtained from specific pathogen free (SPF) pigs. These pigs have lower gamma globulin levels than pigs in the "real" world, and this probably reduces the "noise" (nonspecific binding of gamma globulin) level significantly. Since positives are determined by an arbitrary multiple of the noise level of control pigs, it is desirable to use a control sample that better represents field pigs. Consequently, we have chosen to utilize a commercial pool of pig serum collected at a slaughter plant as our negative control.

On retesting the 70 sera which had previously been in error using the information mentioned above, only three sera were found to be in disagreement with computer printout results. There were two false positives and one false negative (serum neutralization (SN) titer 1:16). The ELA screening dilution was 1:20. The serum bank will be repeated blind during the FY 1976.

IV. TRICHINOSIS ACTIVITIES

Trichinosis is the prototype parasitic disease for adaptation to ELA methodology. ELA appears to offer adequate sensitivity for detecting critical

levels (0.1 to 1.0 cysts/g of diaphragm) of trichina cysts. The testing of numerous "negative" (by digestion) sera does indicate that we presently may have a false positive problem. We believe that reagent purification will yield an eventual solution to this problem.

A. Double Blind Test of Swine Sera for T. spiralis Antibodies by SAFA and ELA Disc Tests

In July 1974, 85 sera were randomly coded by number by two individuals and then tested by the ELA and the soluble antigen fluorescent antibody (SAFA) tests, using the second set of identifying numbers. The sera were sent to us by Dr. W. J. Zimmerman, Veterinary Medical Research Institute, College of Veterinary Medicine, Ames, Iowa, and were part of an experiment reported in an earlier publication.[1] The SAFA and ELA tests were carried out as described earlier.[1,2] The results of the tests are shown in Table III. Results of the SAFA and ELA test differed for 13 of the 85 sera. Twelve of these differences were for pre-immune sera or sera from the early phases of infection, i.e., days 7, 14, or 17 post-infection, and reflect the somewhat greater sensitivity of the ELA test as compared with the SAFA test. On or after day 21, there was one false negative for ELA; 2 for SAFA. There were 2 false positives for ELA; none for SAFA.

B. Adams Farm Sera Tested by SAFA and ELA Microtiter Tray Test[3]

In November 1974, 141 sera collected from Adams Farm, known to be heavily infested with T. spiralis, were sent to us by Drs. Paul Spencer and Frank Adams, Bureau of Animal Health, Illinois Department of Agriculture, Springfield, IL. The sera were identified to us by number only. Digestion data on the animals from which the sera were collected were sent to us after our serological results were transmitted to Dr. Spencer.

1. Comparison of ELA and SAFA Results. One hundred forty-one sera, collected from 141 individual pigs, were tested by both the ELA and SAFA tests. One hundred twenty-eight were positive, 7± and 6 negative by the SAFA test. One hundred thirty-three were positive, 7± and 1 negative by the ELA test. Table IV shows the identifying number of the animals classified either negative or ± by either of the two tests. The two tests identified the same sera as being negative or ±. All of these animals were negative by the digestion test. The large number of ± or negative sera by SAFA, which are positive by ELA, presumably reflects the greater sensitivity of the ELA test.

2. Comparison of Serological Results with Digestion Data. Digestion of tissue from animals corresponding to the serum samples was done in the laboratory of Dr. Robert S. Isenstein, Agriculture Research Service, Beltsville, MD, and sent to us by Drs. Spencer and Adams, after receipt of our serological results.

Fifty-five of the 141 animals were negative by the digestion method. Of these 55, 48 were serologically positive or ± by the SAFA test, and 54 positive or ± by the ELA test. Since 28 of the 55 negative digestion results were based on digestion of only 20 to 25 grams of diaphragm, and the rest from 50 to 120 grams, it is possible that digestion of larger amounts of material might have revealed the presence of very small numbers of larvae in some, if not most, of the digestion-negative, serologically positive animals. All of the animals found positive by digestion were positive by the ELA test, and all but 2, which were ±, were positive SAFA. There was no correlation between the numerical readout of either the ELA or SAFA test and the number of cysts per gram found by the digestion method.

C. ELA Microtiter Tray Test on SPF Swine Sera

In December of 1974, sera from 63 SPF pigs were tested by the ELA Microtiter Tray Test. A total of 285 sera, from 2 to 7 samples per pig, were tested.

TABLE IV

COMPARISON OF ELA AND SAFA NEGATIVES AND ± RESULTS

Date of Serum Collection	ELA Result	SAFA Result
6-27-73	Neg 301[a] ± 100, 501	Neg = 100, 301, 501
7-20-73	± 335	Neg 335 ± 111[c], 201[c], 711[c], 801[c], 901[c]
7-12-73	± 302[b], 303, 311	± 303, 311
7-17-73	± 321	Neg 321, 323[c]
Total	Neg = 1 ± = 7	Neg = 6 ± = 7

[a]Pig identification numbers, all negative by digestion

[b]302 called positive by SAFA

[c]Called positive by ELA

Figure 4 shows the distribution of the means for each of the 63 animals. The average of these means was 34.55 ELA readout units, and the standard deviation of the means 10.78. Based on these data for SPF pigs, a positive value for the test would be about 80 units at 99% confidence level.

D. SAFA and ELA Microtiter Tray Test on Gnotobiotic Swine Sera

In March 1975, Dr. E. H. Bohl, Ohio Agriculture Research and Development Center, Wooster, OH, sent us 17 serum samples taken from gnotobiotic swine and 13 samples from conventional* pigs raised in his laboratory.

Both of the tests gave very low readings for the gnotobiotic pigs. The range for the ELA test is shown in Fig. 4. The mean for ELA was 7, s.d. = 3.16. The ELA mean for the conventional pigs was 58.58, s.d. = 25.01. It was apparent that the sera from gnotobiotic swine were "cleaner" in both the ELA and SAFA test than sera from either SPF or conventional pigs.

E. ELA Microtiter Tray Test on Packinghouse Sera

In January 1975, Dr. Calvin Campbell, USDA, APHIS, VS, State Epidemiologist, P. O. Box 464, Albuquerque, NM, 87103, collected for us 48 whole blood samples from a group of pigs slaughtered at Schwartzman's packinghouse in Albuquerque, NM. Our purpose was to establish that the ELA test could be run on whole, oxalated blood instead of serum and to get some idea of what problems might be encountered in a large-scale field test planned for later in the year at Nashville, TN. Results of our tests showed that whole oxalated blood diluted 1:5 gave the same ELA results as plasma diluted 1:10. For these tests, a serological "positive" was defined as a serum (diluted 1:10) which gave an ELA readout of 100 or more. With this definition, 10 of the 48 samples tested were called positive. In view of the unexpected finding of 21% positives, 4 more collections were made in Albuquerque in the months of February, March, April, and May 1975. Serological results are shown in Table V. Distribution of the ELA reading for the 205 animals in the first 4 groups are shown in Fig. 4. Readings for the 7 May collection were not included in the figure because the test conditions had been changed somewhat in an attempt to reduce the apparent number of "positives."

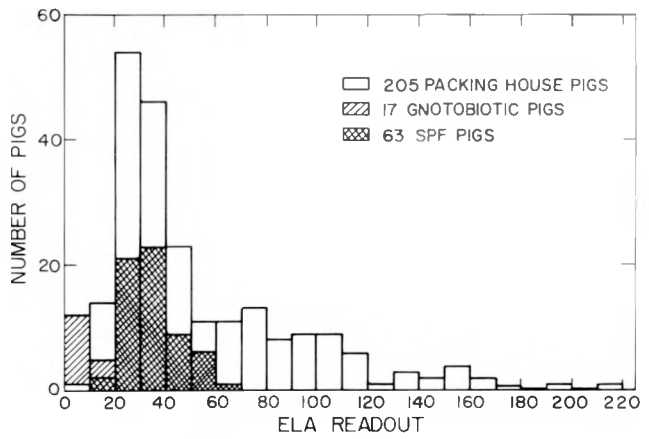

Fig. 4. Histogram of ELA readings of sera from 3 groups of presumed negative pigs. ELA readout for packinghouse pigs ranged from 0 to 220, for gnotobiotic pigs from 0 to 20, and for SPF pigs from 10 to 70.

Possible explanations for the overall 15% positive rate in the packinghouse sera are as follows:

1. Serological positives are due to presence of specific antibodies to T. spiralis.
2. Serological positives are due to presence of antibodies to some other organism(s) which cross-react with our T. spiralis antigen.
3. Serological positives are due to presence of some nonspecific serum factor(s) which binds to our T. spiralis antigen preparation.

Keeping in mind that any or all of the above explanations may apply to any given positive serum,

TABLE V

ELA TEST ON PACKINGHOUSE SWINE SERA

Collection Data	# of Animals Tested	# of Sera "Positive" by ELA Test	%
1-21-75	48	10	21
2-11-75	46	11	24
3-12-75	51	3	6
4-16-75	60	5	8
5-07-75	60	11	17
TOTAL	265	40	15

*These sera were from pigs described as "conventional" by Dr. Bohl. As they were identified by herd, they have been included in the non-SPF, farm raised group in Fig. 4.

but proceeding on the assumption that most if not all of the positives we have seen are due to the same single cause; preliminary experiments have been done in an attempt to distinguish between these possibilities.

The following results are relevant to explanation #1 above. The strongest evidence for the packinghouse serological positives resulting from presence of specific antibodies to T. spiralis would be the finding of T. spiralis larvae in tissues of the slaughtered pigs. On 7 May whole diaphragms, as well as sera, were collected from the 60 pigs slaughtered. The complete diaphragms from the 11 serologically positive pigs were sent to Dr. Robert D. Furrow's Laboratory, Agricultural Research Center, Building 318-A, Beltsville, MD, 20705, where they were found to be negative for T. spiralis larvae by digestion. This negative finding is equivocal because in an experiment done with Dr. W. J. Zimmerman, it was shown that digestion of as much as 900 grams of tissue failed to detect larvae in a pig which had been fed 100 larvae and was serologically positive.

Evidence suggesting that the packinghouse positives are not due to specific antibodies to T. spiralis is the fact that those sera which have been tested fail to show a precipitin line in agar double diffusion tests against the antigen and fail to absorb precipitin activity from the antigen, as judged by an agar diffusion test; however, these results could be due to presence in the sera of specific antibody at levels below the sensitivity of these tests.

The packinghouse positive sera which have been examined behave similarly to known positive T. spiralis sera with respect to the following conditions:

(1) No activity is lost upon storage for 6 weeks at -20°C, 4°C, or room temperature, upon heating at 56°C for 20 minutes, or upon dialysis against saline at pH 8 for 24 hours.

(2) Precipitation of lipoprotein from the sera by dextran sulfate and $CaCl_2$ does not affect activity in the ELA test.

(3) Activity in the ELA test is precipitated by 35% saturation with $(NH_4)_2SO_4$.

With respect to cross reactions as an explanation for the packinghouse positives, the ELA microtiter tray test is negative for pooled sera from four swine parasitized by Ascarids, Trichuris, Strongyloides, and Oesophagostomum, and a serum sample from a feral boar infested with Stephanurus. Both of these samples were sent to us by Dr. D. E. Zinter, MPIP, APHIS, USDA, Scientific Services, Cotton Annex, Beltsville, MD, 20705.

At present a nonspecific serum protein explanation seems most attractive, particularly as we have evidence that some of the packinghouse positives give negative ELA results when highly purified conjugates are used in the test. Our efforts in the coming year will be directed toward fractionation of sera in an attempt to identify, with some degree of certainty, what component(s) are responsible for the packinghouse positives, followed by fractionation and purification of our T. spiralis antigen preparation and purification of the enzyme-labeled conjugates.

F. Lymphocyte Stimulation Test on Swine Lymphocytes

A probe study to determine whether the lymphocyte stimulation test, utilizing the flow microfluorometer can be used to detect swine lymphocytes sensitized by antigens from T. spiralis has been initiated. In March 1975, an experiment was carried out using blood samples from three T. spiralis parasitized swine and one uninfected control blood, sent to us by Dr. W. J. Zimmerman. The test procedures developed by Dr. L. S. Cram, of this Laboratory, for testing bovine lymphocytes for sensitization to tuberculosis (PPD) antigen were followed. Results of this first experiment were negative. Further efforts to adapt this test for detection of T. spiralis sensitized lymphocytes are planned.

V. SWINE BRUCELLOSIS

Swine brucellosis is one of the diseases which the USDA personnel believe to be important in connection with the multiple disease screening concept for ELA slaughter plant utilization.

VSL is in the process of both creating a serum bank and evaluating various Brucella antigens so that a blind testing of the efficacy of ELA for swine brucellosis can be determined. In the meantime, Dr. Paul Spencer (Illinois Department of Agriculture) and Dr. Arthur Starkey (Oscar Meyer Packing Company) have sent a few Brucella culture positive swine sera for ELA evaluation. The results are presented in

TABLE VI

ELA RESULTS ON BRUCELLA POSITIVE PACKINGHOUSE SWINE[a]

Sample	ELA Reading[b]	Signal-to-Noise Ratio[c]	Result[d]
Standard Negative Pool	9	1.0	Negative
16-0133	20	1.9	Positive
20-0133	61	5.8	Positive
21-0133	92	8.8	Positive
14-0214	194	18.5	Positive
5-0241	72	6.9	Positive
9-0241	208	19.8	Positive
15-0241	67	6.4	Positive
16-0241	91	8.7	Positive
Packing Plant Negative Pool	12	1.1	Negative

[a] 10 minute serum incubation (1:25 dilution), 5 minute conjugate incubation (1:100 dilution), 20 minute substrate incubation.

[b] Mean of three replicates.

[c] Determined by dividing suspect sample by the reading of the standard negative pool.

[d] A signal-to-noise ratio $\geq$ 1.5 is considered positive.

Table VI. All sera positive for Brucella on bacterial culture were positive by ELA.

VI. SWINE TUBERCULOSIS

LASL is attempting to develop a rapid ELA screening test to determine swine herds infected with either Mycobacterium avium or Mycobacterium bovis. While ELA, in its present stage of development, misses some infected animals, we believe the test to be useful on a herd basis. Our most significant problem at present is a false positive problem, especially in older animals. Reagent development to overcome this problem is actively underway.

A. Experimental Infections

Our early optimistic results were somewhat compromised when we discovered the intradermal injection of purified protein derivative (PPD) will induce pigs to mount a serological response against PPD antigens. (See Table VII.) These results tell us that skin testing of animals will yield false positive ELA results. A new series of experiments were started in nonskin tested animals. Animals were infected with either M. bovis or M. avium. Sera were collected at periodic intervals and were tested by ELA for humoral antibody to tuberculosis antigens. M. bovis infected animals (Table VIII) both became serologically positive by 28 days post-infection. Five of six M. avium infected animals (Table IX) were serologically positive by day 42. However, one of the noninfected control animals also became serologically positive at day 42. An additional 20 control animals were tested against M. avium antigens. Two of the 20 sera were obtained from mature sows; both were serologically positive. Of the 18 sera obtained from younger animals, two were test positive. Therefore, it appears that a significant false positive problem exists. Work during the coming months will be directed towards developing a more specific antigen for the detection of M. avium infection. Elimination of the

TABLE VII

DEVELOPMENT OF POSITIVE ELA SEROLOGY IN NONINFECTED, SKIN TESTED ANIMALS[a]

Animal Identification	Results of ELA Serology[b]		
	Day 0	Day 22	Day 58
314	Neg	Pos	Pos
316	Neg	Pos	Pos
319	Neg	Pos	Pos

[a] 0.1 ml of PPD-A and PPD-B injected intradermally on days 0, 28, and 55.

[b] All sera were tested blind. Minimum probability values for all positive results are 0.001.

TABLE VIII

DEVELOPMENT OF POSITIVE ELA SEROLOGY IN M. bovis INFECTED, NONSKIN TESTED ANIMALS

Animal Identification	M. bovis Inoculum[b]	Results of ELA Serology[a]			
		Day 0	Day 14	Day 28	Day 41
17097	0.005 mg	Neg	Neg	Pos	Pos
17107	0.01 mg	Neg	Pos	Pos	Pos
17098	None	Neg	Neg	Neg	Neg

[a] All sera tested blind. Minimum probability values for all positive results are 0.001.

[b] Net weight of M. bovis culture #887 deposited into posterior pharynx.

TABLE IX

ELA SEROLOGY AT 42 DAYS IN M. avium INFECTED PIGS

Number of Animals	Number ELA Positive[a]
6 infected	5
3 noninfected	1

[a]Minimum P value of 0.01; all positive animals were still serologically positive at day 70.

false positive reactions would enable the use of ELA, at least in a herd test, to detect foci of M. avium infections. The much less frequently encountered M. bovis infections appear to be adequately detectable with currently available reagents. Routine skin testing of swine should be eliminated when ELA becomes a diagnostic tool for detecting swine tuberculosis.

B. Antigen Development

Initial work on TB has included reading some of the vast amount of literature available on the subject. Some of the pertinent points found were:

(1) Bacille Calmette-Guerin (BCG) produces some antigens in common with tubercular antigens
(2) Antigenic material can be isolated from both the bacterial cells and the filtered media.
(3) The long heat treatment during the preparation of PPD antigens possibly alters the antigens.
(4) Immunoelectrophoresis reveals as many as 60 immunoprecipitates, thus a possible 60 antigens.

Various TB antigens were obtained from Drs. D. E. Pietz and R. D. Angus, VSL, Ames, Iowa. The specific activity of these antigens will be obtained when the work on making the ELA test quantitative is completed.

Work is beginning on testing whether impedance measurements are sensitive enough for measuring PPD or antigen stimulation of lymphocytes and also on measuring fluorescinated antigen or PPD binding to lymphocytes.

VII. PROBES AND TRAINING

Probes constitute short studies designed to test the feasibility of applying our diagnostic test or other methodology to selected areas of interest to the USDA. Training of USDA personnel in our procedures is also done from time to time.

TABLE X

ELA DETECTION OF M. avium and M. bovis INFECTIONS IN CATTLE[a]

	42 Days After Infection		70 Days After Infection	
	M. bovis	M. avium	M. bovis	M. avium
Number ELA Positive[b]	6/10	2/8	8/8	7/9

[a]Of 14 control sera collected at various intervals, 1 was ELA positive.
[b]Probability value $\geq$ 0.01.

A. Bovine Tuberculosis

A brief probe was done on 49 selected sera to determine whether ELA could be useful in detecting bovine tuberculosis. These data are summarized in Table X. All M. bovis infections were detected at day 70 after infection, while two M. avium infections were still not detectable at this time. There was one false positive reaction in 14 control sera. All of the animals used in this experiment were skin tested prior to inoculation. At day 42, all of the controls were ELA negative. Whether or not the skin test has an interfering effect on sera collected before 42 days is not known.

In an additional experiment, an attempt was made to differentiate the strain of infection present in the 23 ELA positive animals. To do this, the positive sera were run against the M. avium and M. bovis PPD's. That PPD which gave the highest signal-to-noise ratio was considered the strain of infection. This criteria proved to be correct in 20 of the 23 sera. Therefore, while ELA may miss occasional positive animals, it may be useful as a herd test in cattle (or in swine), and rapid differential diagnosis in known infected herds may be possible. A large number of samples from older noninfected animals will have to be tested before a realistic evaluation of the false positive problem can be determined.

B. Bovine Cysticercosis

Dr. D. E. Zinter, of the USDA's Animal and Plant Health Inspection Service (APHIS), Meat and Poultry Inspection Program (MPIP), spent two weeks at this Laboratory to learn the principles of the ELA test and to test for the ability of the technique to detect antibodies to Cysticercus bovis in infected cattle. These preliminary experiments were successful in that sera from all three infected animals

were ELA positive; two of the three animals were positive by day 35, and the third was positive at day 63 after infection.

C. Meetings

A paper entitled "Serologic Testing with Enzyme-Labeled Antibodies (ELA)" was presented at the 17th Annual Meeting of the American Association of Veterinary Laboratory Diagnosticians, Roanoke, VA, October 13-15, 1974.[4]

D. Other Training

Dr. Rube Harrington, VSL, APHIS, spent approximately eight days at LASL to be trained in ELA techniques.

VIII. AUTOMATION OF SEROLOGICAL TEST

The ELA serological test is currently being performed by hand. While this has been adequate in the past, it will be necessary to automate the processing for large volume testing and greater quality control. The process instrumentation development has been along three lines:

(1) to provide a quantitative readout of the manual processing,

(2) to start the developmental work toward a fully automated system suitable for slaughterhouse and laboratory processing,

(3) to determine some of the requirements of a national disease reporting system of which the serological test will become a component.

A. Readout for Manual Processing

The manual readout instrument provides a digital indication of the sample absorption. The unit consists of five basic parts:

(1) a light source which emits wavelengths near the absorption maximum of the reaction products, (see Fig. 1).

(2) electronics and optical components to stabilize the light output of the light source,

(3) optics to collimate and focus the light from the source,

(4) detectors used for measuring the absorption and stabilizing the light output and compensating for temperature effects,

(5) a digital voltmeter readout of the absorption measuring detector.

The light source is a Hewlett Packard 5082-4958 gallium-phosphide green, light-emitting diode (LED). The light output is feedback-stabilized to maintain a constant light output. A schematic diagram of the circuitry is shown in Fig. 5. D_1 of Fig. 5 is ten parallel-connected CL-4720 current regulator diodes placed in series with the LED to limit the diode current to values less than 50 ma. This prevents destruction of the LED by high current if circuit malfunction should occur. Light from the LED is routed to one of three (United Detector Technology, Inc., PIN 5DP) planar diffused silicon photodiode detectors. The output voltage from this "clamp" detector is held at a preset level, and the control signal from it is used to maintain a constant output light level as the ambient temperature changes. The signal from a second detector is used to compensate for detector dark current from ambient temperature changes. In this configuration, the unit is stable to better than $\pm$ 0.1% over ambient temperature ranges of a few °C. The third detector is used to measure the light transmission.

The optical bench of the instrument is shown in Fig. 6. Two 11 mm diameter F/1.9 lenses were used to collimate the light from the small active elements of the LED through the sample and then refocus this light onto the detector. The output voltage signal (0 - 1 V) from this detector is read with a digital voltmeter.

The printed circuit board layouts are shown in Fig. 7 and to the left of Fig. 8. Two of the instruments are in routine use with the manual processing.

B. Automated Processing

The plan for implementing full process automation is to divide the development up into three stages. The first stage is to automate each of the essential parts of the processing and to work out the problems associated with these separate operations. The next stage is to incorporate semi-automation into sample collection, animal and sample identification, and record keeping. The final stage is to integrate the semi-automated system into a fully automated one, which will have a minimum of operator manipulation and record keeping. The present effort on automation reported here was directed at stage one which is designing, building, and testing preprototype instruments for each of the four main processing steps.

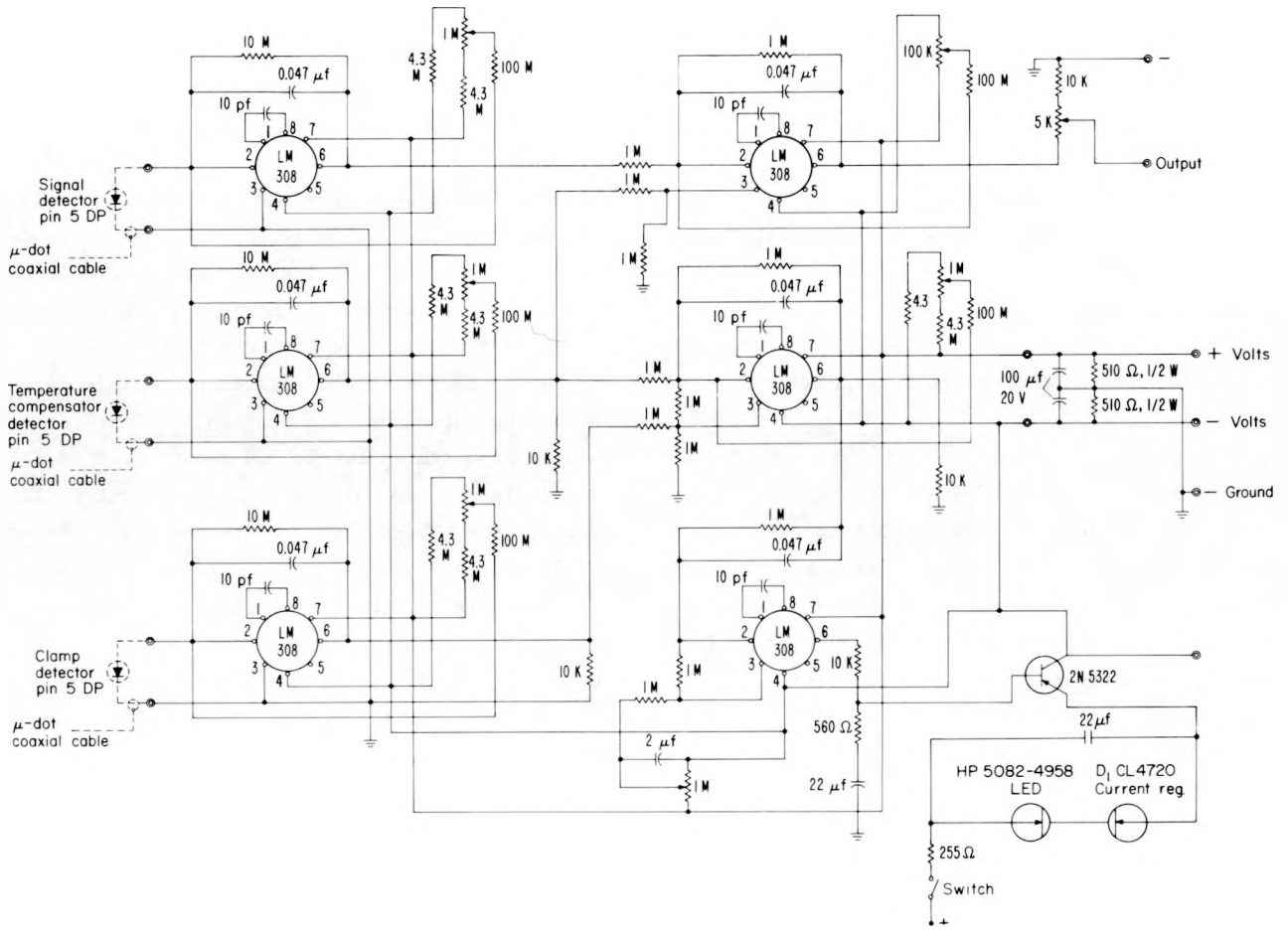

Fig. 5. Circuit diagram of ELA readout.

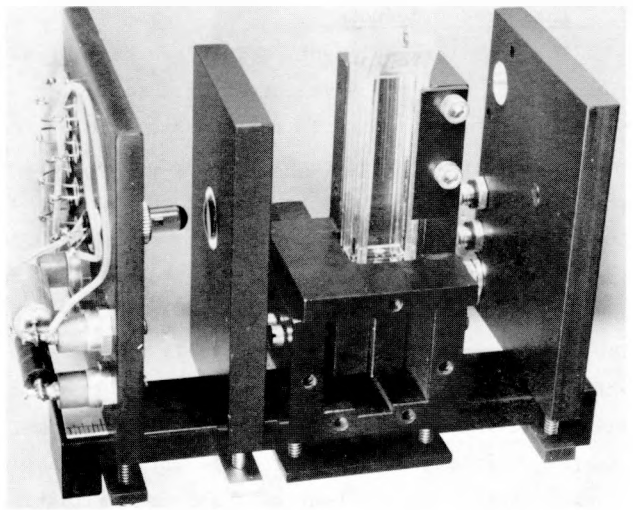

Fig. 6. Optical bench of the ELA readout.

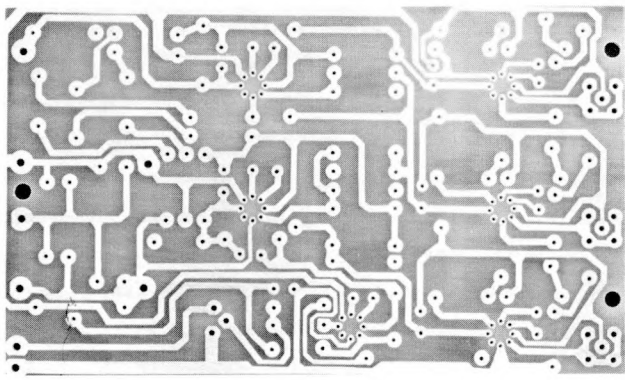

Fig. 7. Printed circuit layout of ELA readout.

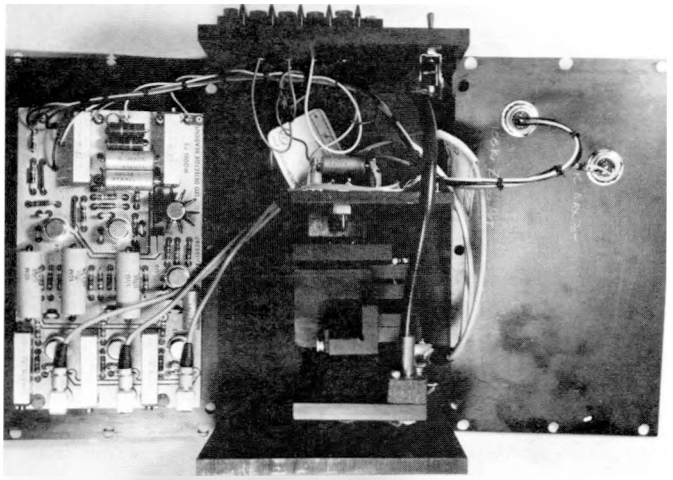

Fig. 8. Partially exploded view of ELA readout. The optical bench of Fig. 6 is in the center, and the topside of the circuit board shown in Fig. 7 is shown to the left.

1. Automated Sample Transfer. To maintain a fixed incubation time for all samples in the microtiter tray, it is necessary to simultaneously transfer the blood samples to the antigen coated tray. Simultaneous transfer is necessary because of the parallel processing steps that will be incorporated into the prototype instrument. The design parameters for the sample transfer mechanism include automatic cleanup of the system after the sample is transferred and well-to-well volume deviations of less than 5%. The total volume of the transferred sample will be limited to one-half the total volume of the microtiter tray (about 0.2 ml).

The preliminary design of the sample transfer mechanism has been completed. The initial design will use a matrix of pistons to draw the sample from a preloaded microtiter tray into stainless steel tubes. The samples will then be transferred into the coated tray. The drawings for this mechanism should be finalized and its fabrication commenced in September 1975.

2. Automated Wash. The individual wells of the microtiter tray must be washed gently so the bound antigen and antibody molecules are not removed, but turbulent enough so the excess materials that are present are removed. The present manual technique for performing the wash step indicates that it should be fairly simple for soluble antigens but may pose more of a problem for cell-bound antigens (viruses). Complete removal of the wash solution may be a difficult task. However, as long as the fraction of wash that remains in each well is the same, there is no problem.

Some of the questions that we have studied about the wash cycle are:

(1) Should the wells in the microtiter tray be individually washed or can the entire tray be washed with spray from a single nozzle?

(2) How efficiently must the wash solution be removed before the succeeding step?

(3) What is the best technique for removing the wash solution from the microtiter wells?

(4) What are the agitation or stirring requirements during the wash cycle?

After considering the questions above, it was decided that the individual wells of the tray will be washed by a gentle flow of wash solution; and the excess fluid will be removed by a mechanical equivalent of the manual operation of shaking the upside-down tray. Adjustments will be made available to optimize the flow rates and mechanical motions.

The wash mechanism is in the early design stages. Drawings should be finalized and fabrication should be started during September 1975.

3. Automated Reagent Addition. The reagent addition steps in the ELA-test procedure involves the addition of about 0.2 ml to each well. By using small-diameter orifices, these additions could be performed in easily regulated time intervals of a few seconds. In some of the studies a #27 hypodermic needle was used for the flow orifice. The points were ground from these needles and their tips were carefully smoothed and reamed. The needles were then carefully cleaned and tested. The flow-rate data that were obtained during these tests are shown in Figs. 9 and 10. Figure 9 shows the flow rate through these orifices versus pressure. A flow rate of 0.1 ml/s is obtained at a pressure of 35.5 kPa (5.1 psi). The flow rate from each individual orifice can be calibrated to a predetermined value by adjusting the length of the cylindrical section of each needle (Fig. 10).

The incorporation of this technique into the automation scheme, because of its simplicity, is quite appealing. However, subsequent tests have indicated that if the cleanup procedure is not complete, precipitation onto the walls of the needle could occur and drastically change the flow rates.

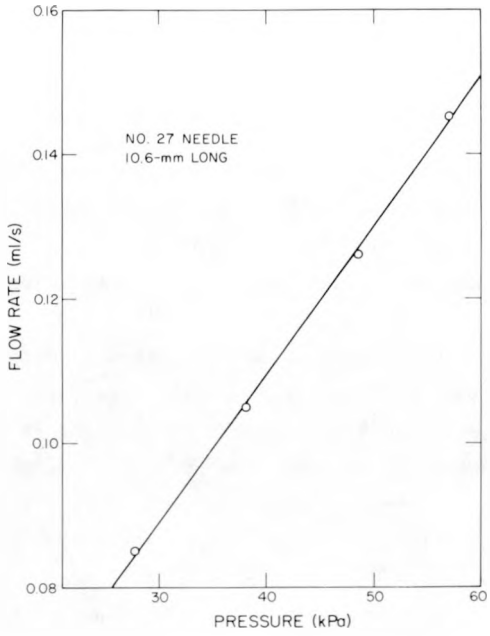

Fig. 9. Plot of flow rate vs pressure of a hypodermic needle.

It is felt that these problems can be solved, and the final design for the reagent addition mechanism will probably use techniques similar to these.

4. Automated Readout. The final step of the ELA-test procedure is to measure the light transmission of the substrate fluid. An instrument that will automatically perform this measurement and record the data for each of the 96 wells of the microtiter tray is being designed. The mechanical portion of the instrument has been designed and is about 50% assembled. The control unit will be checked out when the mechanical portion is complete. While the other elements of automation were designed for a 24-well array, the readout was designed for all 96 wells because the problems were not significantly greater for the full array as compared to a partial array.

An X-Y Table is being designed to hold and position the microtiter trays for readout. The X-Y Table will provide linear motions in the X and Y directions. Mechanical indents are used to precisely position the tray. A binary readout is provided to indicate the position of the tray. This position data is fed to the control unit where it is used to move the tray and is recorded as part of the output record.

The electronic control unit, designated as Model 74 ELA Readout Control, (shown in Fig. 11) will sequence through all 96 samples of a microtiter tray (or any rectangular array less than the full 8 x 12 array). The sequence control can be either fully automatic or manual. The sequence for measuring the light transmission characteristic for each well is (1) position a sample under the read head, (2) apply a vacuum to the read head to clear out any residual liquid, (3) lower the read head into the sample well, (4) apply a vacuum and draw the sample up into a chamber for the absorption measurement, (5) after a settling time, convert the analog output of the measuring instrument to a digital form for display and record the results of the measurement (coordinate information and identification are also recorded to identify the sample), (6) rinse the read head to avoid cross contamination to the next sample, (7) lift the read head. This procedure is repeated for

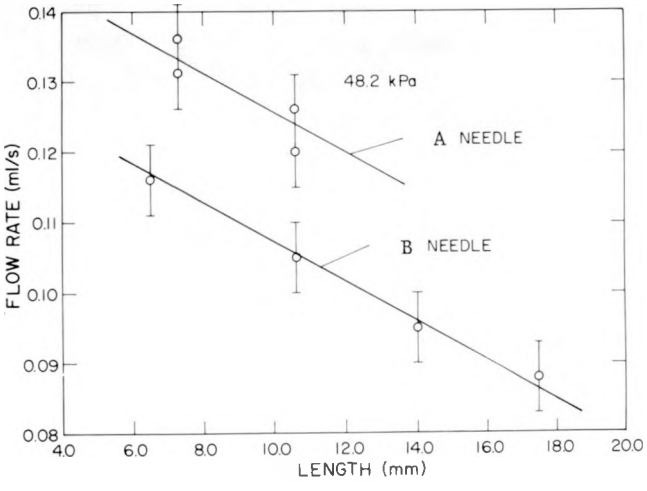

Fig. 10. Flow rate vs length of 2 needle types.

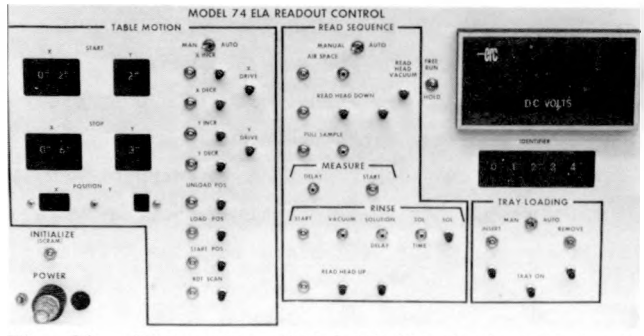

Fig. 11. Front view, Model 74 ELA Readout Control.

each of the designated cells. After the entire tray is read, the table returns to the unload position. The sequence may be interrupted at any time, and the mechanism will revert to a "safe" condition. The option of manual operation is necessary because it is needed for setup and adjustment.

The X-Y Table on which the tray is mounted has the X and Y positions encoded with binary numbers corresponding to 1 through 12 for X and 1 through 8 for Y. The control uses the present position information to make logical decisions for optimum table motion. Any rectangular array of samples may be measured by setting in X and Y start and X and Y stop positions. The control compares the present position with the desired position, and will then move the table with the minimum travel to the load, to the unload, to the start position, or to read through samples position. The operator may assume control in the manual mode and move the table incrementally to select any given sample.

The actual measurement may be sequenced automatically or it may be sequenced by the operator. Normally, the instrument will make one measurement on each sample and hold the information for recording. However, it may be switched to make and display measurements continuously when calibrating the absorption measuring portion of the system. The normal data record for each sample consists of a five-digit identifier, the Y position, the X position, and the results of the absorption measurement. The five-digit identifier (which may contain the tray number) and the X and Y information are used to identify the specific sample.

Assuming a fully automatic process at a later date, provision has been made for automatic loading and unloading of trays to and from the X-Y Table. The load position and unload positions are provided for that purpose. The timing for tray loading is included in the control together with interlock sensing which will be used to insure registration of the tray on the table and proper orientation of the tray

The control unit is completely assembled and wired as shown in Figs. 11 and 12. However, it cannot be considered operational until the mechanical portion is completed and the final operational tests are made. The mechanical portion has been about 50% assembled.

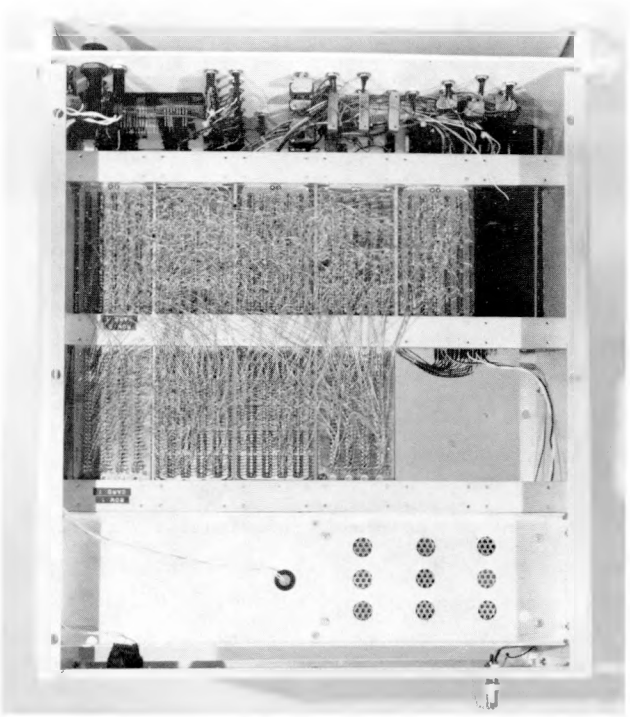

Fig. 12. Bottom view of automatic ELA readout control unit.

IX ANIMAL DISEASE CONTROL

Animal diseases are deleterious to the livestock industry, and in some cases, to the human population. Therefore, the USDA and the livestock industries are engaged in animal disease control and consumer protection programs. Disease control is predicated on detecting the diseased animals, tracing their movements through commerce, establishing quarantines, identifying and testing exposed animals, and suitably disposing of the infected animals. Indemnity payments are made to encourage livestock owners to participate in the control programs.

There is room for improvement in each component of the disease control programs, and LASL scientists have been engaged in work designed to improve the efficiency of some of the individual components. The serological test development reported here has been directed towards providing a fast, low-cost, high thru-put disease screening system for slaughter houses and laboratories. The electronic identification of animals[5] will provide the tie-in between laboratory tests, animal movements through commerce, and the disease indemnity programs. If the industry has high-speed access to accurate records of livestock movements through commerce, then corrective

actions can be initiated to minimize the spread of disease. Electronic identification can provide this capability, if coupled with large-scale computer handling of records. LASL scientists have also been working with the Texas Brucellosis Indemnity program, and have gained experience in handling the very large amounts of data associated with that program. A description of this work is also contained in Reference 5. Thus, we believe that our work in these three areas will result in assisting the USDA and the livestock industry in minimizing the losses associated with animal diseases.

ACKNOWLEDGMENTS

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REFERENCES

1. E. H. Clinard, "Evaluation of the Soluble-Antigen Fluorescent Antibody Test for Antibodies to Trichinella spiralis in Experimentally Infected Swine." Am. J. Vet. 36: 615-618 (1975).

2. G. C. Saunders and M. E. Wilder, "Disease Screening with Enzyme-Labeled Antibodies." J. Infect. Diseases, 129: No. 3, 362-364 (1974).

3. G. C. Saunders and E. H. Clinard, "A Simplified Micromethod of Screening for Antibodies to Disease Agents Using the Indirect Enzyme-Labeled Antibody (ELA) Concept." Submitted to J. of Clinical Microbiology (1975).

4. G. C. Saunders, "Serologic Testing with Enzyme-Labeled Antibodies (ELA)." 17th Annual Proc. of Amer. Assoc. of Veterinary Laboratory Diagnosticians, 311-320 (1974).

5. A. R. Koelle, R. E. Bobbett, J. A. Landt, Wm. M. Sanders, D. M. Holm, and S. W. Depp, "Passive Electronic Identification and Temperature Monitoring System," Los Alamos Scientific Laboratory report LA-6088-PR (1975).

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