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The
MOLECULAR
ANATOMY
(MAN) PROGRAM

Semiannual PROGRESS REPORT

March 1, 1973 to August 31, 1973

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Contract No. W-7405-eng-26

THE MOLECULAR ANATOMY (MAN) PROGRAM

Semiannual Progress Report for the Period

March 1, 1973 to August 31, 1973

Supported by the National Cancer Institute, the
National Institute of General Medical Sciences, the
National Institute of Allergy and Infectious Diseases, and
the U.S. Atomic Energy Commission

Norman G. Anderson
Program Director

DECEMBER 1973

OAK RIDGE NATIONAL LABORATORY
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LIST OF PREVIOUS PUBLICATIONS

The Molecular Anatomy of Cells and Tissues Annual Report, July 1, 1965, to June 30, 1966	ORNL-3978 Special
The Molecular Anatomy of Cells and Tissues Annual Report, July 1, 1966, to June 30, 1967	ORNL-4171 Special
The Molecular Anatomy Program Second Biannual Progress Report, Mar. 1, 1967, to Feb. 29, 1968	ORNL-4273 Special
The Molecular Anatomy Program First Semiannual Progress Report, Mar. 1 to Aug. 31, 1968	ORNL-4319 Special
The Molecular Anatomy Program Second Semiannual Progress Report, Sept. 1, 1968, to Feb. 28, 1969	ORNL-4419 Special
The Molecular Anatomy Program First Semiannual Progress Report, Mar. 1 to Aug. 31, 1969	ORNL-4462 Special
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NOTICE

This report is submitted to fulfill the reporting requirements of the U.S. Atomic Energy Commission and interagency agreements:

1) Joint AEC-NIH/NCI Interagency Agreement 40-388-73 [NCI-CP-73-210]. "Molecular Anatomy Program." *(Work described in Sect. I)*

2) Joint AEC-NIH/NCI Interagency Agreement 40-279-71. "Chemical Profile of Body Fluids." *(Work described in Sect. III.B)*

3) Joint AEC-NIH/NIAID Interagency Agreement 40-194-69. "The Molecular Laboratory, Rockville, Maryland." *(Work described in Sect. II)*

4) Joint AEC-NIH/NIGMS Interagency Agreement 40-95-66. "Body Fluids Analysis." *(Work described in Sect. III.B.)*

5) Joint AEC-NIH/NIGMS Interagency Agreement 40-173-69. "Development of a Fast Clinical Analyzer." *(Work described in Sect. III.A)*

6) AEC Activity No. RX 03 02 00. *(Work described in Sect. V)*

7) AEC Activity No. RX 04 03 00. *(Work described in Sect. IV)*

SUMMARY

The initial observation that fetal cells contain autoantigens which cross-react with antigens of autochthonous tumors has now been amply confirmed in other laboratories, suggesting that we may now get on with basic problems our discoveries have raised.

The preparation of anti-tumor-antigen antibodies and means for detecting and measuring them is continuing. Challenge immunity to tumors in hamsters has been obtained using soluble extracts from SV40 tumor cells injected into splenectomized animals. Immunization with fetal cells is not due to Forssman antigen. Serum from pregnant hamsters will abrogate the cytotoxicity of lymph node cells from SV40 hyperimmune donors in vitro, further implicating normal fetal antigens in both the immunological response to the fetus and the tumor-bearer to the tumor. Experiments designed to determine whether fetal immunization will interrupt leukemogenesis are in progress.

Parainfluenza vaccine has been troubled with loss of immunogenicity on storage. A whole-particle parainfluenza 3 vaccine has been developed which after formalin inactivation, is immunogenic and stable after storage at 37°C for 4 weeks. Similar parainfluenza 1 and 2 vaccines and a trivalent vaccine are now on test. Glycoprotein "spikes" of the respiratory syncytial virus have been isolated and shown to produce both neutralizing (plaque-reducing) and complement-fixing antibodies in guinea pigs. DNA polymerase activity has been found in the Dane particles associated with human hepatitis B antigen. The enzyme appears to be associated with a subpopulation of the Dane-particle cores. A radioimmunoassay and a CF test for these cores have been developed. A large pool of rabbit anti-*a* and anti-*r* subtypes of HBAg have been delivered to the Research Resources Branch of NIAID.

The miniature fast analyzer is now undergoing tests at the NASA Manned Spacecraft Center and in the ORNL Health Division Clinical Laboratories. Twelve procedures have been adapted to the system, of which five (serum glucose, triglyceride, alkaline phosphatase, lactate dehydrogenase, and glutamate-oxaloacetate transaminase) are in routine use. Dynamic loading of samples and reagents allows reagents to be injected and distributed

between cuvets in as little as 1.5 sec at 3000 rpm. The results obtained with discrete and dynamic loading are almost identical. The rotor and optical systems have been modified for either fluorescence or right-angle scatter measurements. The latter has been found useful for monitoring immunoprecipitin reactions. In addition a fluorescence polarization technique is being explored. In routine use the miniature system has been found to yield results of high precision and is being applied to the analysis of age-related trends in ORNL employees. Fluorescamine has been adapted to the monitoring of urinary polyamines separated by high-resolution chromatography. Eight new uv-absorbing constituents from urine have been identified. Current studies on urinary nucleoside excretion have centered on pseudouridine, 1-methylinosine, and N^2-dimethylguanosine.

A "Family Image" has been produced using information accumulated in the course of the ORAU-sponsored gallium group studies. This first synthetic patient image confirms our predictions that such images would provide an objective, quantitative test of the truthfulness of image processing methods. The family image, resembling a patient with Hodgkin's disease, has been used to test the validity of the antiscatter and antipenetration processing. The tests show that the processing produces a very great reduction in the difference between an ideal, noise-free image and the image corresponding to a clinical scan. A visual inspection of the unobtainable ideal image and the processed image shows that both the scatter and the statistical fluctuations have been reduced in effect.

The major emphasis in the MAN Program during the last six months has been on the development of cyclic affinity chromatography systems for the concentration and fractionation of protein mixtures. With suitable immunoabsorbants, with eluting solutions which do not damage the fixed antigens or antibodies or alter the eluted protein, and with automatic recycling equipment, it is possible to (1) remove unwanted antigens from a mixture, (2) concentrate and purify a desired antigen, and (3) remove unwanted antibodies from an antiserum or concentrate and purify a desired antibody. The systems are being applied to two central problems of interest to the AEC: (1) the isolation of human tumor-associated antigens, and (2) the determination of mutation rates in man. Human tumor-associated autoantigens

appear to be reexpressed embryonic or fetal antigens, and the isolation of these is now of central importance. Since some human tumor-associated antigens cross-react with human placental antigens, the five major human placental antigens are being isolated using the Cyclum systems. Alpha-fetoprotein has also been isolated using these methods, as has CEA. The latter has been shown to be chromatographically heterogeneous, and differences in amino acid composition have been demonstrated in the subfractions. Immunosubtractive methods have been developed to remove "all but one" serum proteins, which is then intercompared electrophoretically with an internal standard to detect electrophoretic mutants. A new program has been initiated aimed at improvement of the blood centrifuge.

I. CANCER

I.A. INTRODUCTION

The long-range objective of the study described in this report is to attempt to correlate retrogenic phenomena associated with the presence of fetal antigens in virus-induced tumors of rodents with similar antigens on human cancers. Reexpression of fetal macromolecules in tumor cells may be under the control of cryptic oncodnaviruses or oncornaviruses or may result from carcinogen-stimulated modulation in normal cell regulation. The current state of our understanding of fetal expression in tumors does not permit this research group to be overly concerned with the stimulus for retrogenesis at present, since we are fully occupied with characterizing the antigenic traits of these recrudesced cell products. Rather our current focus is to gather and verify as much information as possible about the basic biological and immunological properties of fetal antigens in anticipation of using these antigens as tools for early detection of neoplastic transformation, whatever the carcinogen. The application of these findings, if developed into useful immunologic tools, in cancer detection and in cancer therapy monitoring is obvious. Perhaps less obvious is the potential usefulness of fetal antigen markers as indices of viral or chemical carcinogenic processes, especially if fetal antigens truly appear universally in cancers.

Some may suggest that several human cancer types are already recognized to have fetal gene products (carcinoembryonic antigen and alpha-fetoprotein, for example), yet initial efforts to make application of these unique products for cancer detection have yielded confusing and sometimes disappointing results. This is specifically the point where our major scientific concern, both theoretically and experimentally, centers. Utilization of "embryonic" markers for cancer detection on a purely empirical basis, without basic information about their autoantigenic traits or the character of the immune response produced by the host to them, will be most difficult and time consuming. Fetal antigens must be exhaustively

characterized now. Considerable information is available to suggest that human fetal antigens will not be unlike rodent fetal antigens, and, indeed there are data to suggest cross-species cross-reactions between rodent and human fetal tissues. Investment of time and funds in the comparative analysis of rodent fetal antigen expression certainly seems justifiable and is the basis for the research described herein. We have recently reviewed the central problems in studying fetal antigens rather extensively [Coggin, J. H., Jr., and N. G. Anderson. "Cancer, Differentiation, and Embryonic Antigens: Some Central Problems." *Advan. Cancer Res.*, in press].

I.B. CORRELATION OF DATA FROM THE MAN PROGRAM STUDY OF FETAL ANTIGENS WITH OTHER RESEARCH REPORTS

It is essential to ask whether the results produced over the past two years from this NCI-supported study of fetal antigens have been suitably verified by other workers in the field and whether our initial data, obtained in the hamster model system, correlate with other model system data and, more importantly, with human cancer systems. The review of such data can best be accomplished by asking first, whether predictions made from the model hamster system can be verified in other rodent systems, and second, whether these findings "fit" current information derived from human cancer studies.

In 1970 we suggested that fetal tissues could evoke humoral antibody and cell-mediated immune responses in both syngeneic and xenogeneic hosts which were cross-reactive with oncodnavirus tumor cells. Our findings were published in parallel with results from Dr. F. Rapp's laboratory which suggested that SV40 tumor cells would react with antibody occurring in maternal hamster serum during normal pregnancy.[1,2] The data were supported by previous reports relating tumor antigens to fetal antigens (Table I.B.1).

The toughest challenge to our initial report was that several other researchers, working from our earliest technical descriptions, had difficulty in eliciting cell-mediated immunity (CMI) with fetal tissues which would prevent tumor formation following challenge in other systems (see Table I.B.2 for typical data from the MAN study). For example, C. C. Ting and his colleagues at NCI could not obtain either humoral antibody or CMI

---

[1] Duff, R. J., and F. Rapp. "Reaction of Serum from Pregnant Hamsters with Surface of Cells Transformed by SV40." *J. Immunol. 105*, 522 (1970).

[2] Coggin, J. H., Jr., K. R. Ambrose, and N. G. Anderson. "Fetal Antigen Capable of Inducing Transplantation Immunity Against SV40 Hamster Tumor Cells." *J. Immunol. 105*, 524 (1970).

Table I.B.1. Summary of Efforts to Detect Embryonic or Fetal Antigens in Model Tumor Systems Prior to 1970

Date	Model	Fetal Antigen Demonstrated	Comment	Reference[a]	Assay	Allogenic	Syngeneic or Inbred
1906	Mice-spontaneous sarcoma (?)	+	Fetal immunization prevented tumor transplantation	Schone, 1906	TR	+	
1962	Rat-human sarcoma H51	+	Cortisone treated rats	Buttle *et al.*, 1962	TR[b]	+	
1964	Mice-chemically induced sarcomas	+	Only detected with non-syngeneic fetus	Buttle *et al.*, 1964; Buttle and Frayn, 1967	TR	+	
1967	Mice-3-MCA sarcomas	+	Moderate to weak protection	Prehn, 1967	TR		+
1968	Mice-polyoma tumor	+	Alloantisera used	Pearson and Freeman, 1968	AS[c]	+	
1968	Mice-polyoma tumor	-	No protection observed against polyoma tumor challenge	Ting, 1968	TR		+
1970	Mice-72 mouse tumors	+	Many tumors cross-react with antifetal sera	Stonehill and Bendich, 1970	AS	+	

Table I.B.1. (Continued)

Date	Model	Fetal Antigen Demonstrated	Comment	Reference	Assay	Allogenic	Syngeneic or Inbred
1970	Pregnant mice-3-MCA sarcomas	+	Pregnant effector cells destroy tumor cells	Brawn, 1970	MC[d]		+
1970	Mice-MTV	-(?)	Attempted to prevent MTV tumors	Blair, 1970	TR		+
1970	SV40-unfertilized mouse ova	+	Alloantisera reacted against SV40 tumor cells	Baranska et al., 1970	AS	+	

[a]References:

Baranska, W. P., P. Koldovsky, and H. Koprowski. *Proc. Natl. Acad. Sci. U.S. 67*, 193 (1970).
Blair, P. B. *Cancer Res. 30*, 1199 (1970).
Brawn, R. J. *Intern. J. Cancer 6*, 245 (1970).
Buttle, G. A. H., J. L. Eperon, and K. Kovacs. *Nature (London) 194*, 730 (1962).
Buttle, G. A. H., J. Eperon, and D. N. Menzies. *Lancet 12*, 14 (1964).
Buttle, G. A. H., and A. Frayn. *Nature 215*, 1495 (1967).
Pearson, G., and G. Freeman. *Cancer Res. 28*, 1665 (1968).
Prehn, R. T. In *Cross-Reacting Antigens and Neoantigens*. (J. J. Trentin, ed.). Baltimore: Williams and Wilkins, 1967, p. 105.
Schöne, G. *Muench. Med. Wochschr. 51*, 1 (1906).
Stonehill, E. H., and A. Bendich. *Nature 228*, 370 (1970).
Ting, R. C. *Nature (London) 217*, 858 (1968).

[b]Tumor rejection assay.

[c]Allogenic serum prepared against mouse fetus, absorbed and tested.

[d]Microcytotoxicity test.

Table I.B.2. Typical Results Demonstrating the Induction of Transplantation Immunity to SV40 Hamster Tumor Cell Challenge Employing Fetal Tissues as Immunogen

Antigen	No. Tumors Detected[a] / No. Animals Challenged	(% Tumors)	Percent Protection[b] Against SV40 Tumor Challenge
Unvaccinated controls	14/15	(93)	control
SV40 tumor cells -- irradiated (5000 R)	0/15	(0)	100
Adult hamster tissue homogenate (5000 R)	15/15	(100)	0
Syngeneic hamster fetal cells:[c]			
9-day gestation (5000 R)	6 15	(40)	53
10-day gestation (5000 R)	5/15	(33)	67
14-day gestation (5000 R)	13/15	(86)	7
9-day gestation (0 R)	13/14	(92)	
14-day gestation (0 R)	9/10	(90)	
Mouse fetal cells BALB/c			
12-day gestation (5000 R)	4/10	(40)	53
Term (5000 R)	5/5	(100)	
Human embryonic kidney cells (5000 R)	7/10	(70)	23
Adult kidney homogenate (5000 R)	9/9	(100)	

[a]Animals challenged with 5 x 10^4 SV40 tumor cells (syngeneic) (50% tumors obtained in controls at day 68).

[b]Test result compared to unvaccinated control in each case to obtain percentage protection.

[c]Viability of cells >40% as determined by the dye-exclusion test using trypan blue.

against SV40-transformed mouse tumors following methods described as similar to our methods.[3] They reported that sera containing antibody against many papovavirus tumors *could not be absorbed* by fetal tissues of several gestational ages. *No* protection against SV40 tumor challenge was noted in mice immunized against irradiated mouse fetal cells. In these and subsequent studies reported by Ting, mice were subjected to x-irradiation prior to challenge so that the highly antigenic SV40 tumor could be used.

In our system autochthonous virus-induced primary tumors were used and we had no data on the capacity of fetal tissue to *potentiate* the rejection of nononcogenic tumors in irradiated recipients. Herberman working with Ting in a rat lymphoma system did detect cytotoxic spleen cells in rats primed with x-irradiated rat fetus.[4] X-irradiation of fetus was required to obtain induction of CMI in a fashion identical to the hamster model.

Since the initial reports from our laboratories, we have further documented the observation that antigens present on fetus could indeed stimulate tumor resistance and active CMI. Information presented in Table I.B.3 outlines essential steps in preparing fetal vaccines collected from a number of our publications.

In 1972, Ting and his colleagues indicated that fetal tissues could indeed elicit antibodies in inbred mice which were cross-reactive with SV40 and other tumor cells, but again, they could not detect cell-mediated tumor immunity in mice primed with fetus.[5,6] Baldwin reported (in

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[3]Ting, C. C., D. H. Lavrin, G. Shiu, and R. B. Herberman. "Tumor-Specific Cell Surface Antigens in Papova-Virus-Induced Tumors and Their Relationship to Fetal Antigens." *Proc. 1st Conf. and Workshop on Embryonic and Fetal Antigens in Cancer.* USAEC Report CONF-710527, 1971, pp. 223-37.

[4]Herberman, R. B., C. C. Ting, and D. H. Lavrin. "Immune Reactions to Virus-Induced Leukemia in Animals Immunized with Fetal Tissues." *Proc. 1st Conf. and Workshop on Embryonic and Fetal Antigens in Cancer.* USAEC Report CONF-710527, 1971, pp. 259-66.

[5]Ting, C. C., D. H. Lavrin, and R. B. Herberman. "Antibodies to Fetal Antigens." *Embryonic and Fetal Antigens in Cancer, Vol. 2.* USAEC Report CONF-720208, 1972, pp. 111-15.

[6]Ting, C. C., D. H. Lavrin, G. Shiu, and R. B. Herberman. "Expression of Fetal Antigens in Cancer Cells." *Proc. Natl. Acad. Sci. 69*, 1664 (1972).

Table I.B.3. General Guidelines on Preparation and Use of "Fetal" Vaccine for Achieving Protection Against Oncodnavirus Tumors *in Hamsters*

1. Employ time-mated females where *fetal age* is accurately known (±0.5 days).
2. We have not used trypsin to disperse fetal tissues, but tissues have been disaggregated by passing through small-orifice needles.
3. Use disaggregated cell preparations of fetus with *high viability* to achieve maximal immunogenicity (40-60%).
4. Use fetus from *primiparous* females only (fetus from multiparous females seems to be coated with IgG which "masks" immunogenicity).
5. Give multiple intraperitoneal injections of fetus.
6. X-irradiate the fetus to prevent differentiation of the tissue in vivo and to prevent embryoma induction in vaccinees.
7. Keep the time from fetal harvest to injection *less* than *1 hour* to avoid plasma membrane antigen degradation by hydrolytic enzymes released from cells destroyed during fetal disaggregation.
8. Avoid the use of passaged tissue-cultured fetal cells, which undergo apparent in vitro differentiation.
9. Select the correct age fetus for maximal immunogenicity by actually testing each day of fetal age possible (do not make an empirical selection of a given fetal age for convenience sake); for example, in the SV40 model system, fetal antigen expression is "silenced" between 10.5 and 11.0 days.
10. Use the *most sensitive challenge assay possible* (e.g., TPD_{50}) until all factors above are determined.

several places) that rat tumors indeed had fetal antigens reactive with maternal antibody, and, more pertinent to our consideration of cellular immunity, he observed that these tumors were selectively destroyed by *maternal lymphocytes* in in vitro tests; yet, Baldwin could not mimic the response by injection of rat fetal cells into adult recipients.[7,8] In reviewing the methodology of both Ting and Herberman, we noted several deviations employed by these workers from the preparative procedure that we had described for the hamster system to achieve active CMI following vaccination with fetus. Technical difficulties seemed the obvious explanation since clearly maternal effector cells from pregnant rats (Baldwin) or mice (Brawn) or hamsters (Dierlam et al.) killed the respective tumors with ease in in vitro tests. The difficulty was not in the absence of fetal antigens from the tumors but rather, we suspected, in the technical skill of preparing living fetal cells of suitable quality and in the selection of proper methods to achieve sensitizations to the test vaccines.

A case in point -- As many as 4-18 immunizations with *living non-oncogenic SV40 tumor cells* were required to elicit antibody depicting the presence of TSTA-associated antigens using the radioimmunoassay in the SV40 mouse assay system of Ting and co-workers. Fetal vaccines, however, were tested for CMI induction following only three injections with 5 million x-irradiated fetal cells in their comparisons.

Frankly, we began to worry that direct immunization with fetal tissues might not be possible following these several negative reports. But subsequent careful studies performed elsewhere gave renewed encouragement.

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[7] Baldwin, R. W., D. Glaves, and M. V. Pimm. In *Progress in Immunology, Vol. I.* (B. Amos, ed.). New York: Academic, 1971, p. 907.

[8] Baldwin, R. W., D. Glaves, and B. M. Vose. "Fetal Antigen Expression on Chemically Induced Neoplasms." *Embryonic and Fetal Antigens in Cancer, Vol. 2.* USAEC Report CONF-720208, 1972, p. 193-98.

Girardi and Reppucci[9] confirmed our initial observation that irradiated fetal cells could prevent the occurrence of *autochthonous* SV40 neoplasms and further demonstrated that fetuses from multiparous pregnant donors, used by several previous workers, were not antigenic. This observation was confirmed.[10]

Since the "rocky" beginning, many workers have demonstrated that fetal tissues *can* promote tumor rejection and specifically CMI using methods which we described. A summary of these latest findings is presented in Table I.B.4.

The clear demonstration that fetal antigens induced CMI in rats, mice, hamsters, and guinea pigs should quiet the storm of alarm generated by the few negative reports which occurred. We do not, at this juncture, suggest that all rodent cancers possess fetal antigens capable of promoting tumor rejection; as in all biological situations, there are always a few exceptions. We cannot, however, spend time with the rare exception, but must concern ourselves with the remaining majority (see Table I.B.5).

What is clear from our studies is that fetal antigens represent a new and distinct class of antigens which require somewhat different handling and immunization principles from typical antigens which most immunologists encounter. We admit, even after three years of study, considerable ignorance in this area. First, we are dealing with "autoantigens" and we know precious little about these. Second, we are dealing with antigens which "nature" may have intended to use as "survival antigens" in vitro which are recrudesced in cancer. What is clearly missing is detailed, quantitative information about these antigens; this void is the core of our current research effort.

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[9] Girardi, A. J., and P. Reppucci. "The Relationship of Hamster Fetal Antigen to SV40 Tumor-Specific Transplantation Antigen." *Embryonic and Fetal Antigens in Cancer, Vol. 2.* USAEC Report CONF-720208, 1972, pp. 167-70.

[10] Girardi, A. J., P. Reppucci, P. Dierlam, W. Rutala, and J. H. Coggin, Jr. "Prevention of Simian Virus 40 Tumors by Hamster Fetal Tissue: Influence of Parity Status of Donor Females on Immunogenicity of Fetal Tissue and on Immune Cell Cytotoxicity." *Proc. Natl. Acad. Sci. 70*, 183-86 (1973).

Table I.B.4. Reported Observations of Induction of Tumor Immunity Following Direct Immunization with Fetal Tissue

System	Tumor Immunity Induced Against:	Fetal Vaccine Effective in		Route of Vaccination	Assay	Reference[a]
		Males	Females			
Mouse	QUA	ND[b]	+	SC[c]	Direct challenge	Bendich et al., 1973
Mouse	MCA-10	ND	+	?	Direct challenge, Serum cytotoxicity with ^{125}Iododeoxy-uridine	LeMevel and Wells, 1973
Mouse	PCT	+	ND	IP[d]	Spleen colony assay (FCFU[e])	Salinas et al., 1972
Rat	MCA-R	+	+	?	Direct challenge	Grant and Wells, 1973
Guinea pig	MCA-A, MCA-25	+	+	ID (+CFA)[f] IP	DTHR[g] Direct challenge	Grant et al., 1973

[a]References:

Bendich, A., E. Borenfreund, and E. H. Stonehill. *J. Immunol. 111*, 285 (1973).
Grant, J., S. Ladisch, and S. A. Wells. *Cancer* (in press).
Grant, J., and S. A. Wells. *Surgical Forum* (in press).
LeMevel, B. P., and S. A. Wells. *Nature New Biol. 244*, 183 (1973).
Salinas, F. A., J. A. Smith, and M. G. Hanna, Jr. *Nature 240*, 41 (1973).

[b]Not done.

[c]Subcutaneous injection.

[d]Intraperitoneal injection.

[e]Fetal colony forming unit.

[f]Intradermal injection (complete Freund's adjuvant).

[g]Delayed-type hypersensitivity reactions.

Table I.B.5. Tumors in Syngeneic Systems Recognized to Possess Embryonic or Fetal Antigens

Animal Model: Tumor Type	Inducer or Tumor Origin (No. Different Tumors Tested)	Antigen Detected	
		In Vivo[a]	In Vitro[b]
Hamster:			
Sarcoma	Adenovirus 7-T[c]	+	+
	Adenovirus 31	+	+
	DMBA-T	+	+
	Moloney sarcoma virus-T	+	NT
Carcinoma	3-Methylcholanthrene (MCA)-T (2)	+	+
	Polyoma	±	+
	SV40-T (3)	+	+
	Spontaneous sarcoma-T	+	NT
Lymphoma	Gross virus	NT	+
Rat:			
Sarcoma	3-MCA-T (13)	+(?)	+
Carcinoma	DMBA (4)	?	+
Lymphoma	Gross		+

Table I.B.5. (Continued)

Animal Model: Tumor Type	Inducer or Tumor Origin (No. Different Tumors Tested)	Antigen Detected	
		In Vivo[a]	In Vitro[b]
Mouse:			
Sarcoma	SV40 MKS-A-T	+	+
Leukemia	FMR-Induced-T	+	+
Lymphoma	?-Spontaneous	NT	+
Carcinoma	3 MCA-T		
Plasma cell tumor	Spontaneous-T	+	+
Guinea pig:			
Carcinoma	3-MCA (4)-T		
	DMBA (2)-T	NT	
Sarcoma	Liposarcoma-3-MCA-T		NT
	Osteogenic sarcoma-T		NT

[a]Immunity induced by fetal immunization, detected by transplantation resistance test or interruption of oncogenesis.

[b]Immunity detected in vitro using antibody or cell-mediated tests.

[c]Tumor transplant.

Table I.B.5. (Continued)

References: List compiled from findings in our own laboratory and from the following sources:

Baldwin, R. W., D. Glaves, and M. V. Pimm. In *Progress in Immunology*. Vol. 1, 1971, p. 907.

Bendich, A., E. Borenfreund, and E. H. Stonehill. *J. Immunol. 111*, 285 (1973).

Borsos, T., and E. J. Leonard. *Embryonic and Fetal Antigens in Cancer, Vol. 2.* USAEC Report CONF-720207, 1972, p. 206.

Brawn, R. J. *Intern. J. Cancer 6*, 245 (1970).

Girardi, A. J., P. Repucci, P. Dierlam, W. Rutala, and J. H. Coggin, Jr. *Proc. Natl. Acad. Sci. U. S. 70*, 183 (1973).

Grant, J., S. Ladisch, and S. A. Wells. *Cancer* (in press).

Hanna, M. G., Jr., R. W. Tennant, J. M. Yuhas, N. K. Clapp. B. L. Batzing, and M. J. Snodgrass. *Cancer Res. 32*, 2226 (1972).

Herberman, R. B., C. C. Ting, and D. H. Lavrin. *Proc. 1st Conf. and Workshop on Embryonic and Fetal Antigens in Cancer.* USAEC Report CONF-710527, 1971, p. 259.

I.C. IMMEDIATE WORKSCOPE

This project is currently charged with the investigation of five objectives relating to fetal antigens on cancer cells of rodents. These include:

Project I. Determine the character of humoral and cellular immune reactions to fetal antigens.

Project II. Determine the immunologic and biologic relationships between fetal antigens and TSTA.

Project III. Determine the immunologic relationship between fetal antigens and other common or native antigens (organ-specific antigens, Forssman, etc.) and characterize the fetal antigens to a limited extent biochemically.

Project IV. Explain the corollary between maternal sensitization to fetus in utero to the disparity detected in females primed directly with syngeneic fetus.

Project V. Evaluate the ability of fetal tissues and their antigens to prevent spontaneous neoplasms. A pilot experiment is currently planned.

I.C.1. Project I. Humoral and Cellular Immunity to Fetal Antigens

In this study our major concern is with parenteral (ip) and subcutaneous (sc) immunization with fetal tissues in the hamster and the mouse. Our entire program now uses only inbred hamsters and mice to establish results for publication. In the past we had used the unusual random-bred but syngeneic LVG hamster which has *never* shown any histoincompatibility differences by skin graft testing (primary or second set), tumor transplantation interference, isotopic antiglobulin testing, cytostatic antibody testing, immunofluorescence testing, in vitro microcytotoxicity testing, and lymphocyte stimulation testing using tissue and organ suspensions,

extracts, and homogenates, or skin grafts, or whole embryo homogenates. The system is further blessed with the absence of H-Y loci which cause detectable problems in other systems. The LVG strain is used for initial large-scale screens or pilot projects against autochthonous SV40 tumors because of the high litter yield, high pregnancy rate, high degree of fetal thriving, disease resistance, low indigenous C virus contamination, and the inexpensive aspect of research using these animals. If LVG hamsters have histocompatibility differences, they cannot be detected by any classic or new test of which we are aware. To avoid confusion and criticism, all basic observations are confirmed in inbred LSH hamsters, naturally at greater cost.

A summary of results to date is given in Table I.C.1. Adult male hamsters primed directly with *whole* cell homogenates of hamster fetus (x irradiated) of the indicated gestation period (ip) were observed for tumor resistance to SV40 challenge or to autochthonous SV40 tumor induction in a multiplicity of tests using the many indicated test parameters. It is clear that the fetal antigen in question is antigenically silenced by the 11th day of gestation. Immunization [three injections (sc)] with whole fetal cells does not elicit antibody detectable by the radioimmunoassay, although the donors showed up to 80% protection against tumor challenge. Soluble fetal extracts and soluble tumor extracts were very potent inducers of antibody detected by the IAT-radioimmune assay, but animals yielding these sera had *no* tumor immunity.

Our current efforts center on two principal areas.

I.C.1.a. Isolation of Cytostatic (C) Antibody (IgG) Induced by SV40 Tumor Cells and by Fetal Tissues

Purified fractions of IgG were prepared from large pools of hamster serum rasied against SV40 tumor cells with predetermined levels of C antibody. IgG fractions from placebo-immunized donors served as the control. Serum fractions (IgM, IgG, and albumin) were tested for cytostatic potential in vitro: 0.25 ml of test serum was admixed with 0.25 ml of SV40 target cells (50,000 live cells) and incubated for 18 hr at 37°C in a shaker bath. Viable cell counts were determined in the Cytograf (Biophysics/Systems) following the addition of trypan blue (0.1 ml of 0.5% solution).

Table I.C.1. The Phasing of Fetal Antigens as Demonstrated by Various Immunologic Tests Against SV40 Tumor Cells Following Direct Parenteral Immunization

Immune Reaction	Immunization with X-Irradiated, Dye-Excluding Cells of Fetal Age (days post coitus)							
	9	10	11	12	13	14	15	Birth
Induction of cytostatic antibody[a]	+	+	-	-	-	-	-	-
Induction of Ab by radioimmunoassay[b]	-	-	-	-	-	-	-	-
Induction of cell-mediated immunity								
in vivo[c]	+	+	-	-	-	-	-	-
in vitro[d]	+	+	-	-	-	-	-	-
Induction of tumor resistance[e]	+	+	-	-	-	-	-	-
Interruption of oncogenesis[f]	+	+	-	-	-	-	-	-

The techniques employed to obtain these data are described in detail in the references cited below.

[a]By diffusion chambers [Ambrose, K. R., N. G. Anderson, and J. H. Coggin, Jr. *Proc. 1st Conf. and Workshop on Embryonic and Fetal Antigens in Cancer.* USAEC Report CONF-710527, 1971, p. 281] and in vitro assay.

[b]By IAT test [Ting, C. C., R. B. Herberman, D. H. Lavrin, and G. Shiu. *Proc. 1st Conf. and Workshop on Embryonic and Fetal Antigens in Cancer.* USAEC Report CONF-710527, 1971, p. 223].

[c]By adoptive transfer test [Dierlam, P., N. G. Anderson, and J. H. Coggin, Jr. *Proc. 1st Conf. and Workshop on Embryonic and Fetal Antigens in Cancer.* USAEC Report CONF-710527, 1971, p. 203].

[d]By microcytotoxicity test - colony inhibition [Dierlam, P., N. G. Anderson, and J. H. Coggin, Jr. *Proc. 1st Conf. and Workshop on Embryonic and Fetal Antigens in Cancer.* USAEC Report CONF-710527, 1971, p. 203].

[e]By transplantation immunity [Coggin, J. H., Jr., K. R. Ambrose, and N. G. Anderson. *J. Immunol. 105*, 524 (1970)].

[f]By prevention of SV40 tumor induction in neonatally infected animals [Coggin, J. H., Jr., K. R. Ambrose, B. B. Bellomy, and N. G. Anderson. *J. Immunol. 107*, 526 (1971)].

Target cells in normal IgG or calf serum go through one to two generations. Cells treated with IgG from tumor-immune donors were unable to divide under identical conditions of test but were not killed (Table I.C.2).

Table I.C.2. Detection of Cytostatic Antibody in Serum Fractions from SV40-Tumor-Resistant Hamsters

	Percent Cytostasis[a]			
	Whole Serum	IgM Peak	IgG	Albumin
Normal serum	Control	Control	Control	Control
Immune serum	19	0	49	0

[a]From 10 tubes averaged. (Cytostasis determined from comparison of cell counts between normal serum or its fractions with respective immune serum fractions x 100.) The significance of cytostasis shown was $p = <0.001$ in both situations by Student's t test.

These data indicate the usefulness of the in vitro assay for monitoring C antibody with pure IgG (confirmed by Ouchterlony test). Note also that the isolation procedure yielded a 2.5-fold increase in activity when the IgG fraction was diluted to original volume.

Specificity studies using heterologous target cells are currently in progress, but no difficulty is anticipated since pretesting of serum pools prior to fractionation have demonstrated the desired specificity (using adenovirus tumor target cells). Also currently under test are the cross-absorption studies with homologous and heterologous tumors and with fetus (described briefly in Sect. I.C.2).

Since fetal tissues are strong inducers of C antibody, large batches of pretested serum from hamsters sensitized to syngeneic and xenogeneic fetus of different fetal age are being prepared. Sample donors are tested for tumor immunity. These sera and their IgG fractions will be tested for cytostatic activity in vitro and for specificity by cross-absorption studies.

I.C.1.b. Localization of Fetal Antigens in Fetal Tissues

We are presently preparing sera against various fetal tissues isolated from embryos and fetuses to use in the C antibody assay in vitro. Two approaches are being employed. Dissected parts (liver, brain, etc.) are used to make cell suspensions for *immunization* and for *absorption* studies. Because the yield of such tissues from many fetuses is very small, "subtractive" experiments are also under way. Fetus cell suspensions are prepared from the carcas -- minus the organ (liver, lung, etc.) removed for the above studies; these suspensions are injected into syngeneic recipients in an effort to determine the need for specific organs to raise the desired immunoglobulin. This work has been in progress for 3 months now. Testing and absorption of sera will begin in October. In these studies sera are also tested for "IAT" antibody, and the donors are evaluated for tumor resistance both in vitro and in vivo (see Table I.C.1 for test parameters).

I.C.2. Project II. Relationship of Fetal Antigen to Tumor-Specific Transplantation Antigen

Very early after the recognition that tumor cells possessed fetal antigens a dilemma became apparent concerning the relationship of fetal antigens to tumor-specific transplantation antigens (TSTA). If fetus of the hamster contained common antigen protective against SV40, adenovirus, and Moloney sarcoma tumors, why would this antigen, when present on the tumors, not confer cross-protection between them? One explanation suggested by Ting et al. using the radioimmunoassay for antibody against tumor cell surface antigen (TASA) in the mouse model was that there existed many fetal antigens, all present on fetal cells but individually present on tumors. Hence, each tumor might possess a distinctive display of fetal antigens not present on other tumors.

Another possibility is that TSTA may represent modulated antigenic determinants directed by cellular DNA information but the synthesis of which is regulated by the transforming virus genome. Since the regulatory action of each virus might be distinctive and the modulation at variance in each tumor phenotype, the fetal antigens might not confer cross-protection when tumor cells are used as antigen. Sufficient reactive sites might be present on *fetus* in unmodulated form and thereby confer cross-protection to many tumors. Supportive, indirect data exist for either situation, but the essential data must come from studies of *cell-mediated immunity* using purified antigens from tumors and fetus.

Ting and collaborators have presented suggestive data that the determinants for specific binding of IAT antibody against TASA correlate with TATA (tumor-associated transplantation antigen = TSTA?) in the mouse. We have extensively evaluated this relationship in the hamster in hopes of being able to use the radioimmunoassay to conduct specificity studies to permit discrimination between TSTA (TATA) and fetal antigen. We had some initial doubts about the usefulness of the radioimmunoassay in an autochthonous virus-induced tumor hamster model. Ting had used sera raised by exhaustive immunization regimens with nontumorigenic, in vitro transformed SV40 tumors. We believed that these SV40-transformed cells were not tumorigenic because of their extreme antigenicity, and we recognized that it might be difficult to detect similar antibody in our system where malignant, in vivo derived neoplasms were used. Nevertheless, we developed the IAT-radioimmune methodology, with the assistance of Dr. Ting, who was most helpful in adapting the assay to the hamster model. Following some 8 months of intensive study, we have obtained the following information for the SV40 inbred hamster system:

1. Using SV40 tumor cells (x-irradiated) as immunogen, with up to six immunizations, we cannot detect suitable specific antibody by the radioimmunoassay to conduct the specificity studies described by Ting in the mouse system. For example, using sera at low dilution (1:2 to 1:16) we can detect sera which yield AR ratios of 2.44 (required for significant evaluation of the IAT procedure); however, following absorption of these reactive sera with

SV40 or heterologous tumor (e.g., adenovirus tumor or MSV tumor) or normal tissues, we obtain only 10-15% reductions with SV40 tumor over nonspecific absorption with normal tissues or heterologous tumor; this is not out of range for the experimental error encountered in repeated tests with many sera.

2. Tumor resistance induced by x-irradiated tumor cells does not correlate with the presence or absence of TASA immunoglobulin detected by the radioimmunoassay. Specifically, hamsters immunized with one to three injections of x-irradiated tumor cells *do not* have detectable antibody against TASA, but they are highly immune to SV40 tumor challenge. These hamsters do have C antibody.

3. Using soluble extracts of SV40, adenovirus tumors, or fetus, we detect extremely *high titers* of immunoglobulin, with the radioimmunoassay obtaining AR ratios of 15 to 25 with different sera. Absorption of TASA is specific when induced with SV40 tumor. These extracts do not induce transplantation resistance. Preliminary data in fact suggest that antibody detected with the IAT procedure reflects enhancement of tumor growth and may be a most useful test for antibody to soluble antigens from tumor or fetus.

We continue our efforts to employ more specific sera for this assay. Ting recently suggested that his assay might detect IgM. We are reevaluating our sera with antiglobulin against IgM and IgG to test for results similar to those observed by Ting.

We propose that Ting may be inducing immunoglobulin reactive with both TSTA and fetal antigen present on SV40 tumors of the mouse. The procedure requires maximum dilution (usually more than 1:100) of the serum to an end point still yielding an AR ratio of 2.0. The highest titer of Ting's best sera using nononcogenic SV40 tumor cells is AR = 4. If we plot the relationship of AR to serum dilution (Fig. I.C.1) and assume that the sera

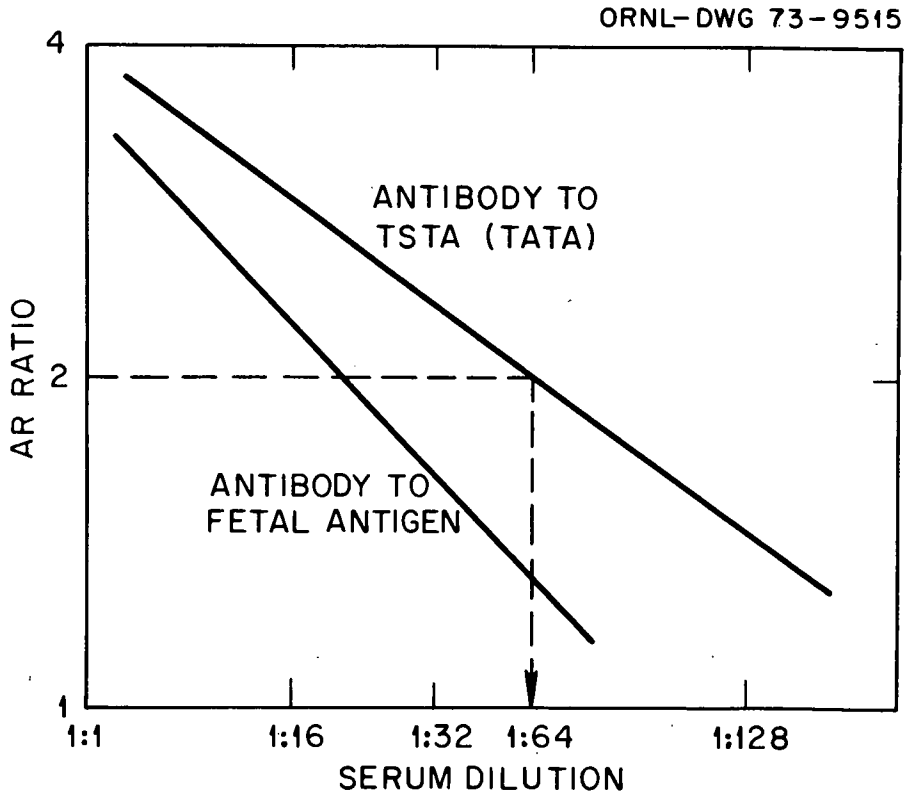

Fig. I.C.1. An Alternate Explanation Concerning the Apparent Specificity of TSTA and Fetal Antigen Detected by the Radioimmunoassay Following Absorption with SV40 Mouse Tumor Cells. Antibody to both determinants may be present at dilutions of 1:16, but antibody to fetal antigen could not be detected at a higher dilution (1:32), since the test end point is arbitrarily set at 2.0.

used have antibodies to *both* TSTA and fetal antigen on the SV40 tumor cells used as vaccine, we can see how the specificity that has been reported for the mouse model might *not* actually be indicative of monospecific antibody but might rather be a dilution effect. Results plotted are not unlike titers reported by Ting. At dilutions of 1:32 the cross-reactive antibody to fetal determinants present in his antitumor sera would not be detectable, whereas absorption studies would indicate specificity for TASA.

What actually might be measured is a quantitative difference in the *amount* of antibody, and the results actually say little about the true specificity of the antibody present in the serum tested. One could predict that the antisera of mice raised in response to repeated injections of SV40 mouse tumors might actually contain many antibodies against a variety of antigenic determinants. Thus the SV40 mouse tumor might actually contain immunogenic determinants for fetal antigen as well as for TSTA. No cross-reactive antibodies in antitumor sera were reported following fetal absorption.[1]

We fortunately can proceed with our efforts to discriminate between TSTA and fetal antigen using C antibody which was present in hamsters with tumor immunity. Now that we have been able to perform the test for C antibody in vitro, we can proceed with the absorption studies described in Sect. I.C.1.

In addition we are now able to obtain tumor immunity in hamsters immunized with soluble extracts from SV40 tumor cells when immunization is coupled with *splenectomy*. The capacity to use soluble cell-free antigens for specificity studies should be most helpful. We are currently concluding similar studies with soluble fetal extracts essential for comparative physiochemical and immunological studies described in the proposal.

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[1]Ting, C. C., D. H. Lavrin, and R. B. Herberman. "Antibodies to Fetal Antigens." *Embryonic and Fetal Antigens in Cancer, Vol. 2.* USAEC Report CONF-720208, 1972, pp. 111-15.

I.C.3. Project III. Relationship of Fetal Antigens to Other Antigens

Prior to June 1973 we concluded an evaluation of the possibility that fetal antigen might be Forssman antigen. The results submitted for publication are summarized in Table I.C.3. Briefly, the results showed that Forssman antigen was present on the surface of SV40 tumor cells and on fetal cells, but unlike fetal antigen, Forssman antigen increased after the 11th day of gestation. We concluded that fetal antigen capable of inducing tumor resistance is not a Forssman antigen.

We are currently seeking to determine the relatedness of organ-specific antigens to fetal antigens. The possibility that specific tissues in the embryo harbor fetal antigens is being tested using the spleen colony assay modified and recently used by Hanna to measure the cross-reaction of tumor-associated antigens and fetal antigens. These experiments are currently in progress as described in the proposal.

I.C.4. Project IV. Materno-Fetal Relationships and Tumor Immunology

I.C.4.a. Female Responsiveness to Fetal Antigen

We observed that female hamsters primed parenterally with fetal tissues had no cell-mediated immunity but developed a significant immunoglobulin response.[2] We are presently evaluating the effects of hormones and splenectomy on direct immunization with fetus in efforts to promote CMI. Since CMI is induced transiently in association with maternal exposure to fetal tissue in utero, we were hopeful that immunization by different

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[2] Dierlam, P., N. G. Anderson, and J. H. Coggin, Jr. "Immunization Against Tumors with Fetal Antigens: Detection of Immunity by the Colony Inhibition Test and by Adoptive Transfer." *Proc. 1st Conf. and Workshop on Embryonic and Fetal Antigens in Cancer.* USAEC Report CONF-710527, 1971, pp. 203-15.

Table I.C.3. Comparison of Forssman Antigen with Fetal Antigen and TSTA

Antigens Tested	Antigen Present on Surface of:						Induction of Tumor Immunity
	10-Day Hamster Fetal Cells	12-Day Hamster Fetal Cells	14-Day Hamster Fetal Cells	12-Week-Old Hamster Cells	Sheep Erythrocytes	SV40 Tumor Cells	
Forssman[b]	+	+	+	±[c]	+	+	-
Tumor-specific transplantation antigen[a,d]	-	-	-	-	-	+	+
Fetal or phase specific[a,d]	+	-	-	-	-	+	+

Techniques employed to obtain these data can be found in the references cited below.

[a]Coggin, J. H., Jr., K. R. Ambrose, B. B. Bellomy, and N. G. Anderson. *J. Immunol. 107*(2), 526 (1971).

[b]Burger, M. M. *Nature New Biol. 231*, 125 (1971).

[c]Noonan, K. D., and M. M. Burger. In *Proc. 1st Conf. and Workshop on Embryonic and Fetal Antigens in Cancer*, (N. G. Anderson and J. H. Coggin, Jr., eds.). USAEC Report CONF-710527, 1971, pp. 59-69.

[d]Coggin, J. H., Jr., K. R. Ambrose, and N. G. Anderson. *J. Immunol. 105*(2), 524 (1970).

routes might achieve sensitization. Grant, Bendich, Wells, and Lemevel have independently obtained significant CMI and tumor immunity in mice and guinea pigs with subcutaneous immunization with fetal tissues (5000 R x ray). We are adopting their methods in addition to continuing the procedures described in the workscope of the proposal.

I.C.4.b. Ovarian Ligation and Tumor Immunity

In another study we observed that ligation of the ovary resulted in tumor immunity in hamsters. A detailed accounting of this work will be presented in a paper which has been submitted to *Science* [S. G. Winslow, N. G. Anderson, and J. H. Coggin, Jr., "Tumor Immunity Following Simple Ovarian Ligation in Hamsters"]. We are currently initiating a study to determine whether ovarian ligation and subsequent autosensitization to fetal antigens on the unfertilized ova can serve to interrupt SV40 oncogenesis in neonatally infected hamsters and in high incidence leukemia mice.

I.C.4.c. Cross-Reactivity Between Blocking Factors in Pregnant and in Tumor-Bearing Hamsters

The immune response of hamsters to autochthonous SV40-induced sarcomas has been evaluated by a number of immunologic techniques in an effort to discover the time course of humoral and cell-mediated responses to tumor surface antigens. These studies, largely completed, are summarized in Table I.C.4. Cytotoxic lymph node cells (LNCs) could be detected in hamsters prior to the appearance of SV40 tumors and, with somewhat diminishing effectiveness, after virus tumors appeared. Identical batches of LNCs or PECs would not confer *passive* protection. In a paper submitted to the *Journal of Immunology* [J. H. Coggin, Jr., P. J. Dierlam, C. Y. Whitley, and N. G. Anderson, "Specific Activation of Cytotoxicity of Lymphocytes In Vitro from Tumor-Bearing Hamsters"] data related to the removal of substances from LNCs from tumor-bearing animals are discussed in detail, with some probable explanations for this discrepancy. Blocking sera were obtained after 15 weeks. Results demonstrate that efferent blocking at the

Table I.C.4. Comparative Study of Immunologic Responses Against SV40 Target Cells in the SV40-Neonate Model

Percent Hamsters with:	Weeks Post Infection with SV40					
	0	5	10	15	20	25
SV40-Induced Sarcomas	0	0	5	30	65	100
C Antibody[a]	-	+	+	+	-	-
S Antibody[b]	-	-	-	-	-	-
IAT Antibody[c]	-	-	-	-	-	-
Cytotoxic LNCs[d]	-	+	+	+	±	±
Protective LNCs[e]	-	±	±	-	-	-
Challenge Immunity[f]	-	-	+	+	±	±
Blocking Serum	-	-	-	+	+	+

[a]Cytostatic antibody.

[b]Surface antibody.

[c]Antibody detected by radioimmune test.

[d]Detected in microcytotoxicity test using SV40 target cells.

[e]Detected in adoptive transfer test.

[f]Concomitant immunity.

LNCs could be detected in multiparous but not primiparous hamsters, showing that similar factors appear with parity following maternal sensitization to fetal antigens in utero (Fig. I.C.2). Studies are presently under way to confirm an earlier observation in our laboratory that serum from pregnant hamsters will abrogate the cytotoxicity of LNCs from SV40 hyperimmune donors in vitro. If this finding is confirmed, the implications of fetal antigens to tumor cell survival in vivo become most provocative.

I.C.5. Project V. The Capacity of Fetal Antigens to Prevent Spontaneous Leukemia in AKR Mice

The protocol described in the proposal is being implemented in the pilot experiment. In addition, we are presently evaluating the capacity of fetal tissues to reduce the oncogenicity of SV40 injected *intravenously* into weanling hamsters. Such infection yields a spectrum of neoplasms including lymphomas, carcinomas, and leukemias.

In the initial proposal concerning fetal immunization against spontaneous neoplasms of mice, we suggested the use of a well-characterized strain of mice (BALB/c) with a relatively high incidence of solid neoplasms (74%) and 49% lymphoma/leukemia to be held for 545 days (mean death time). Also, we anticipated the use of the B_6C_3 hybrid having only 56% solid tumors and 32% lymphoma/leukemia for 870 days of holding.

The cost of such experiments was high, and it was decided to attempt initial studies in AKR mice. Hays[3] recently reviewed the immune responses of the AKR mouse and pointed toward some interesting problems that may arise in our effort to conduct the planned study. AKRs apparently are capable of normal humoral responses, although some aspects of the cell-mediated immune system may be defective (macrophage migration, graft vs host), but their capacity to reject tumors or skin grafts seems intact. They are chronically infected with Gross leukemia viruses and react weakly

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[3]Hays, E. B. In *Virus Tumorigenesis and Immunogenesis*. (W. S. Ceglowski and H. Friedman, eds.). New York: Academic, 1973, p. 321.

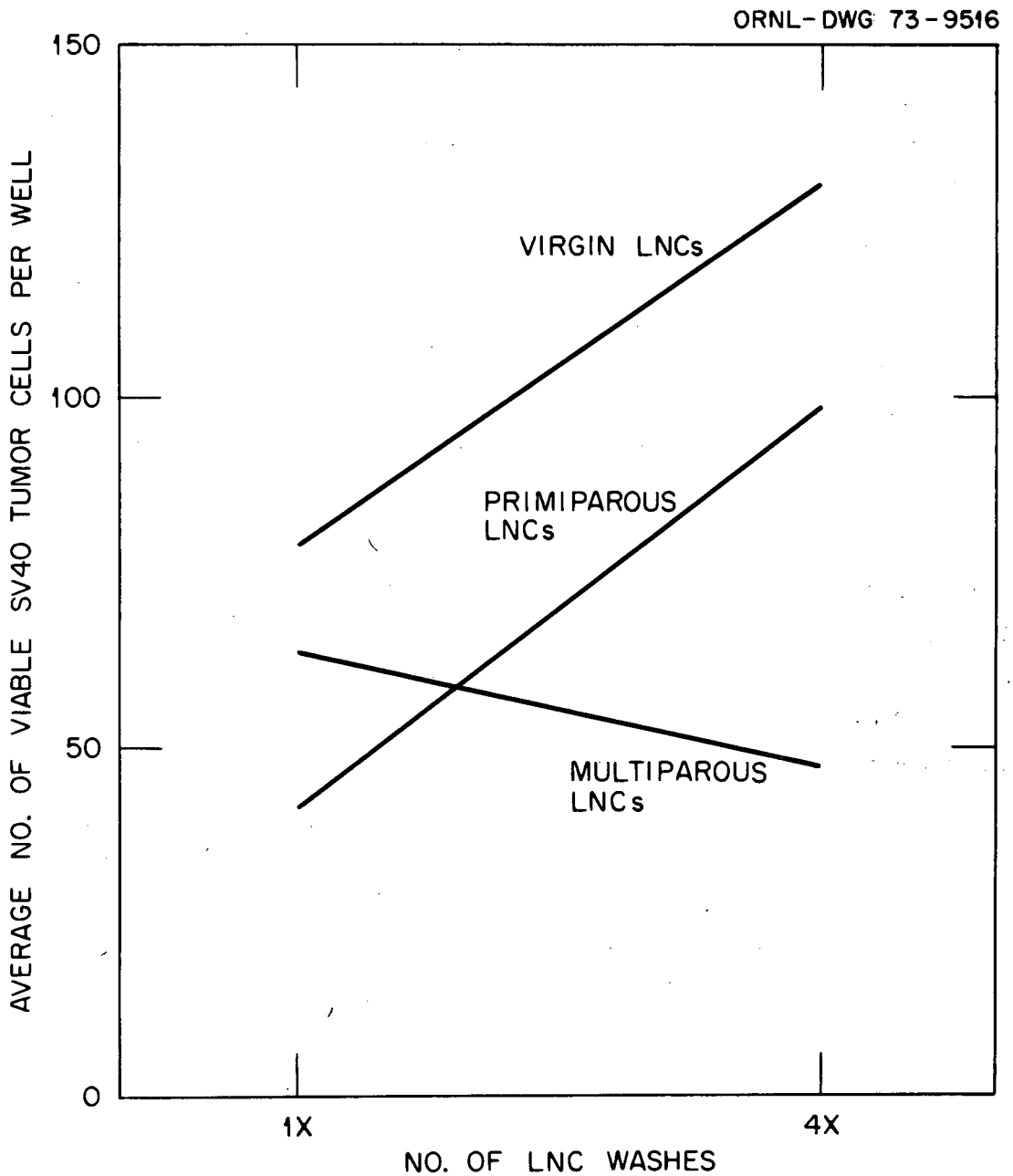

Fig. I.C.2. The Effect of Washing Lymph Node Cells in a Microcytotoxicity Assay Using an SV40 Tumor Target Cell.

to cell surface antigens associated with Gross virus maturation, dying after 6 months with lymphoma related to high virus titers in the thymus and lymphatics. Embryonic cells contain gs-1 and virus. Injection of syngeneic Gross-transformed cells repeatedly into AKR mice can produce antibody cytotoxic for the tumor cells, and inoculation of *allogeneic* Gross virus lymphoma cells improves survival of AKR mice challenged with syngeneic lymphoma cells. *We do not know whether immunization against spontaneous lymphomas in AKR mice can be achieved with syngeneic tumors and, if not, perhaps we will be expecting too much of fetal antigens to induce protection.*

Neuraminidase treatment of lymphoma cells has resulted in an enhanced response to lymphoma by *delaying* tumor appearance by *several weeks*. Hays suggests that CMI to Gross-virus-associated tumor surface antigens may be defective but amenable to stimulation, hence we have included adjuvants in some of our trial fetal vaccines. Hays states "No firm evidence exists for a cellular immune response to Gross virus or its *induced* antigens in AKR mice." Metcalf and Wahren[4] reported that lymphocytes cytotoxic for *embryo* cells could be found in preleukemia AKR prior to 3 weeks to 9 months of age. Thus, mouse embryo cells must have Gross-virus-associated *cell* antigens capable of promoting (reacting with?) CMI. We were forced to test xenogeneic hamster fetus as vaccine and allogenic BALB/c embryos to attempt to avoid this experimental artifact. Hays suggests that adoptive transfer experiments may be possible in young AKR mice using Gross virus lymphoma cells. This system will be used in the near future to further search for cross-reactions between natural mouse leukemia and fetal antigens.

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[4]Metcalf, D., and B. Wahren. In *Immunity and Tolerance in Oncogenesis*, Vol. II. (L. Severi and R. J. Huebner, eds.). 1970, 959-73.

II. INFECTIOUS DISEASES

II.A. INACTIVATED PARAINFLUENZA VACCINE

The clinical evaluation of inactivated parainfluenza vaccine in the past has been troubled by the immunogenic stability of one or more of the type-specific viral antigens. For this reason the Infectious Diseases Branch of the NIAID requested the Molecular Anatomy Program's Rockville Laboratory to develop a method of producing a purified and stable inactivated trivalent parainfluenza vaccine suitable for use in human clinical trials. In an earlier report we described the preparation of a whole particle parainfluenza 3 vaccine which has now been evaluated for immunogenicity after accelerated stability tests. Based on the stimulation of >80% plaque-reducing antibody in hamsters, this formalin-inactivated vaccine was both immunogenic and stable after storage at 37°C for 4 weeks.

Subsequently, we prepared similar vaccines of parainfluenza 1, 2, and 3 which are presently being evaluated for their immunogenic stability; a trivalent vaccine made from the monovalent pools is also on test.

Hamsters immunized with these vaccines by either intranasal or parenteral inoculation will be challenged with live virus to determine in an animal model whether or not the vaccines protect against infection with homologous virus.

II.B. RESPIRATORY SYNCYTIAL VIRUS (RSV)

Guinea pig antiserum to the glycoprotein "spikes" of RSV was assayed for neutralizing (plaque-reduction) and complement fixing (CF) antibodies. The antiserum was shown to contain a 50% plaque-reduction titer of 1:5120 in the presence of added complement. The CF antibody titered 1:64 against RSV grown in AGMK and <1:2 against the AGMK control. Therefore, the glycoprotein "spikes" of RSV as removed from the purified virus particle by bromelain treatment stimulated both specific neutralizing and CF antibodies. Such antiserum will be most useful in sorting out the various antigenic components of RS. Analysis of double-banded and solubilized RS virus by acrylamide gel electrophoresis (AGE) revealed some 6 or 7 proteins ranging in approximate size from 22 to 74 thousand daltons. We are presently attempting to assign these polypeptides to the various structural components of the virus.

The RNP of RS as isolated from HeLa cells after CsCl banding was analyzed by electron microscopy. The diameter of the RNP was 14.0 nm, with a pitch of 71 Å. The distribution of lengths based on careful measurements of some 150 strands of RS RNP revealed some six populations varying from 0.17 to 1.0 μ.

II.C. HEPATITIS TYPE B ANTIGEN

DNA polymerase activity was detected in each of eight preparations of concentrated hepatitis B antigen (HBAg) rich in Dane particles prepared by high-speed centrifugation of antigen-positive human plasma; such activity was detected in none of seven control samples prepared in the same way from HBAg-negative plasma. The incorporation of ^{3}H-TTP into DNA was dependent on four deoxyribonucleoside triphosphates and $MgCl_2$. Treatment of the concentrated HBAg preparations with NP-40 more than doubled the enzyme activity. Fractionation of the concentrated HBAg preparation in sucrose density gradients after treatment with NP-40 revealed that the enzyme activity appeared within the density range of Dane core antigen, but at a slightly higher density than the average for core antigen. The only particles observed by electron microscopy in this region of the gradient were typical 28-nm cores, suggesting that the DNA polymerase activity was associated with a subpopulation of cores. No DNA polymerase activity was found in purified 22-nm HBAg particles. The DNA product of the reaction remained associated with the 110S core and was not susceptible to DNase digestion when associated with the core. Inhibition of the reaction by actinomycin D and daunomycin suggested that the reaction was dependent on a DNA template associated with the core.

Complement fixation tests and radioimmunoassays for detecting the core of Dane particles were developed. The core was detected in Dane particle concentrates but not in preparations of 22-nm forms of HBAg. Detergent (NP-40) treatment of Dane particles increased the detectability of the core. Antibody to the core was detected following acute type B hepatitis infection and in chronic carriers of HBAg.

A 1000-ml pool of rabbit antiserum to the *adr* subtype of HBAg was delivered to the Research Resources Branch of NIAID. This pool contains anti-*a* and anti-*r* antibodies with no detectable anti-normal serum activity and little or no anti-*d* antibody. This antiserum will be used for the detection of *r* determinants; it can be used at a 1:4 dilution in agar gel diffusion analysis and titers 1:64,000 by passive hemagglutination assay. An additional 2400 ml of comparable serum is being held in bulk storage at the MAN Program for future needs.

II.D. PUBLICATIONS

Author(s)	Title	Publication
Kapikian, A. Z., J. L. Gerin, R. G. Wyatt, T. S. Thornhill, and R. M. Chanock	"Density in Cesium Chloride of the 27 nm '8FIIa' Particle Associated with Acute Infectious Nonbacterial Gastroenteritis: Determination by Ultracentrifugation and Immune Electron Microscopy"	*Proc. Soc. Exptl. Biol. Med.* *142*(3), 874-77 (1973)
Kasel, J. A., R. B. Couch, J. L. Gerin, and J. L. Schulman	"The Effect of Influenza Antineuraminidase Antibody on Virus Neutralization"	*Infection and Immunity* *8*(1), 130 (1973)
Shih, J. W., and J. L. Gerin	"Biochemical Studies of Hepatitis B Antigens (HBAg)"	Am. Soc. for Microbiol., *Abstracts of the Ann. Mtg.*, V 156 (1973)

II.E. ORAL PRESENTATION

Speaker and Coauthor	Title	Place Presented
Shih, J. W., and J. L. Gerin	Biochemical Studies of Hepatitis B Antigen (HBAg)	American Soc. for Microbiol., *Abstr. Ann. Mtg., 1973*, V. 156, May 6-11, 1973

III. BIOTECHNOLOGY

Progress continues on the application of advanced technology to the clinical laboratory sciences. The two major development areas are: (1) the development of fast analytical systems for the rapid, parallel analysis of large numbers of body fluid samples for individual molecular constituents, and (2) the development and use of automated high-resolution analytical systems for the analysis of large numbers of the molecular constituents of body and tissue fluids. In both programs basic studies are being carried out on pertinent separation techniques, data reduction methods, biochemical procedures, etc.; however, in each program specific analytical systems that will ultimately be useful in the clinical or research laboratories are being developed, built, and evaluated.

III.A. FAST ANALYZER DEVELOPMENT

C. A. Burtis, W. F. Johnson, J. C. Mailen, J. B. Overton,
C. D. Scott, L. H. Thacker, T. O. Tiffany, and M. B. Watsky

Fast analytical systems are being developed to provide automated, fast analyses of molecular components in physiologic fluids by use of colorimetric, fluorometric, and sedimentation assays. These systems are primarily based on the GeMSAEC principle and include a multicuvet rotor which rotates through a stationary monitor whose output is processed in "real time" by means of an on-line computer or other data processing system.

III.A.1. Miniature Fast Analyzer

A primary effort has been the development of a miniature analytical system in an effort to decrease the overall size and cost. As shown in Fig. III.A.1, integral components of such a system include a miniature analyzer, several plastic rotors of various designs, an automated sample and reagent loader, and a modified version of a previously developed data printer. Two such systems have been fabricated and are undergoing testing and evaluation in the clinical laboratories of the NASA Manned Spacecraft Center and the ORNL Health Division. Many of the routine clinical assays have been adapted for use with the system, and special emphasis has been placed on operating in the multichemistry analytical mode.

To extend the range of applications, a fluorescence monitor is also being developed for use with the miniaturized system. Clinical tests based on fluorometric techniques are currently being adapted or developed for use with this system.

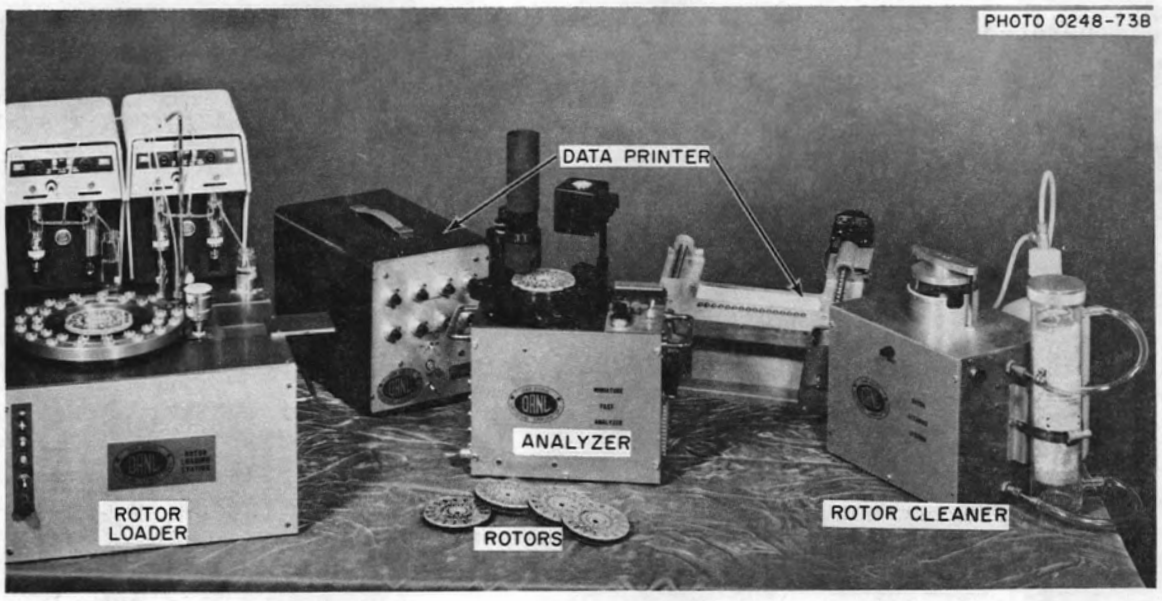

Fig. III.A.1. Miniature Fast Analytical System.

III.A.1.a. Operation and Evaluation

To evaluate the miniature Fast Analyzer under routine laboratory conditions, miniature Fast Analyzer systems have been placed in the clinical laboratories of the NASA Manned Spacecraft Center and the ORNL Health Division.

NASA Evaluation. -- The analytic system shown in Fig. III.A.1 was delivered to the clinical laboratories of the NASA Manned Spacecraft Center, Houston, Texas, on February 1, 1973. The system has been evaluated and operated by personnel of the Northrup Corporation, which is NASA's subcontractor for their clinical facilities. The system has been operating satisfactorily, and plans are now being finalized to consider such a system in the NASA space shuttle.

ORNL Evaluation. -- An analytical system similar to the NASA system shown in Fig. III.A.1 has been placed into operation in the clinical laboratory of the ORNL Health Division. The ORNL system utilizes a computer for data handling, while the NASA system uses a mechanical data printer.

Using the previously described compact computer system,[1] a software package has been developed for routine operation of the system. The computer program has an executive file which initiates the daily operation and, by a series of operator question:response routines, will automatically load the required program into the computer core. An example of the output from the executive program is shown in Fig. III.A.2. Twelve chemical procedures have been adapted for use with the miniature Fast Analyzer, five of which (i.e., serum glucose, triglyceride, alkaline phosphatase, lactate dehydrogenase, and glutamate-oxaloacetate transaminase) are routinely used in the ORNL clinical laboratory.

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[1] *Molecular Anatomy (MAN) Program Semiann. Progr. Rept., Sept. 1, 1972, to Mar. 1, 1973,* ORNL-4867.

ORNL-DWG 73-9517

ORNL HEALTH DIVISION MINIATURE FAST ANALYZER RESULTS

DAY 330 TIME 15 : 50 : 35

QUALITY CONTROL DATA

DAY 330

ASSAY	CONTROL	N	MEAN	SIGMA	C.V.	95% RANGE
GLUCOSE	M-1	84	78.8	3.39	4.31	72.0 - 85.5
	M-2	84	212.6	7.29	3.43	198.0 - 227.2
	PS	84	84.8	3.31	3.90	78.2 - 91.4
TRIGLYCERIDE	STD#1	44	296.0	6.98	2.36	282.0 - 309.9
	STD#2	44	305.6	12.10	3.96	281.4 - 329.8
URIC ACID	M-1	11	9.9	0.26	2.60	9.4 - 10.4
	M-2	11	9.1	0.41	4.47	8.3 - 9.9
	PS	11	5.2	0.35	6.75	4.5 - 5.9
ALK. P'HASE	M-1	40	33.3	2.66	8.00	28.0 - 38.6
	M-2	40	214.9	8.31	3.87	198.3 - 231.5
	PS	40	35.5	4.33	12.20	26.8 - 44.2
LDH-L	M-1	40	48.8	5.26	10.79	38.2 - 59.3
	M-2	40	201.9	12.78	6.33	176.4 - 227.5
	PS	40	50.6	7.01	13.85	36.6 - 64.7
SGOT	M-1	45	18.9	1.85	9.84	15.1 - 22.6
	M-2	45	51.0	2.28	4.48	46.4 - 55.5
	PS	45	7.6	1.44	18.96	4.7 - 10.5

DO YOU WISH TO CALIBRATE A ROTOR (YES OR NO):NO

TO SELECT A CHEMICAL PROCEDURE ENTER ONE OF THE FOLLOWING CODES

CODE	CHEMICAL PROCEDURE
401-	GLUCOSE (FASTING)
402-	GLUCOSE (1 HOUR)
403-	GLUCOSE (2 HOUR)
404-	GLUCOSE (TOLERANCE)
411-	GLUCOSE (RANDOM)
405-	URIC ACID
408-	ALKALINE PHOSPHATASE
409-	SGOT
410-	LDH-L
412-	TRIGLYCERIDES
700-	GLYCEROL CALIBRATION
800-	TERMINATE RUN
900-	RESTART

ENTER DESIRED CODE :401

Fig. III.A.2. Typical Output Obtained from Executive Program Used with the Miniature Fast Analyzer.

III.A.1.b. New Developments

New areas include the development and refinement of techniques for dynamic introduction of liquids into a spinning rotor and the addition of a fluorometric and light-scattering monitor to the optical head of the analyzer.

Dynamic Loading of Liquids. -- Liquids may be introduced into the miniature Fast Analyzer in either a discrete or dynamic mode. In the discrete mode, discrete aliquots of liquids are obtained by means of a pipetting device and transferred and dispensed into their respective cavities in a stationary rotor. The main disadvantages of discrete loading are the relatively high cost of automated instrumentation and the decreased portability of such a loading system.

To circumvent these two disadvantages, the technique of dynamic loading of liquids into a spinning rotor is being developed. Basically the technique consists of dynamically introducing a volume of liquid (reagent or sample) into the centrifugal field generated by the spinning rotor of the Fast Analyzer. The rotor can be designed to apportion the volume of liquid into discrete measured volumes that are automatically transferred into their respective cuvets for reaction initiation, followed by photometric monitoring of the individual reactions.

A dye dilution technique was developed to measure the volume per cuvet obtained by dynamically loading a known volume of liquid into a spinning rotor, and a computer program was written which automatically calculates the dynamically loaded volume per cuvet. A statistical analysis is performed on the calculated volumes and absorbances of the diluted dye solution in cuvets 2-17 to evaluate the splitting performance of a rotor. Using this technique, several of the parameters important in dynamic loading were identified and investigated. They included rotor design and geometry, rotor speed, injection rate, and probe diameter.

A previously described rotor[2] was modified in order to improve its splitting performance. Modifications included (1) removal of plastic curls on the surfaces of the splitting vanes before assembly, (2) increasing the volume capacity of the receiving chambers of the rotor, and (3) machining of a hemispherical cavity into the center of the rotor to allow injection directly onto the faces of the splitting vanes.

Injection probe diameter was found to be a more critical parameter than rotor speed or rate of injection. A decrease in the tip diameter of the injection probe increases splitting performance in a linear manner (Fig. III.A.3). Optimum splitting performance was obtained at a rotor speed of 3000 rpm with an injection time of approximately 1.5 sec. Typical analytical results obtained with dynamic introduction of reagent are shown in Table III.A.1.

Other Monitoring Systems. -- A fluorescence monitor was previously developed for use with the 15-place GeMSAEC Fast Analyzer.[3,4] Two approaches have been taken in transferring this technology to the miniature Fast Analyzer: (1) adaptation of right-angle excitation/emission using specially designed rotors with end windows in each cuvet normal to the standard UVT top window, and (2) frontal excitation/emission as previously developed for the 15-place GeMSAEC Fast Analyzer.

A right-angle rotor and detection system developed for the miniature analyzer is shown in Fig. III.A.3. This rotor can be used for both fluorescence and light-scattering measurements. It was designed specifically for low-level fluorescence measurements where a right-angle rotor would have the advantage of reduced reflection and light scatter over a frontal

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[2]Burtis, C. A., J. C. Mailen, W. F. Johnson, C. D. Scott, T. O. Tiffany, and N. G. Anderson. "Development of a Miniature Fast Analyzer." *Clin. Chem. 18*, 753 (1972).

[3]Tiffany, T. O., C. A. Burtis, J. C. Mailen, and L. H. Thacker. "Dynamic Multicuvette Fluorometer-Spectrophotometer Based on the GeMSAEC Fast Analyzer Principle." *Anal. Chem. 45*, 1716 (1973).

[4]Tiffany, T. O., M. B. Watsky, C. A. Burtis, and L. H. Thacker. "Fluorometric Fast Analyzer: Some Applications to Fluorescence Measurements in Clinical Chemistry." *Clin. Chem. 19*, 871 (1973).

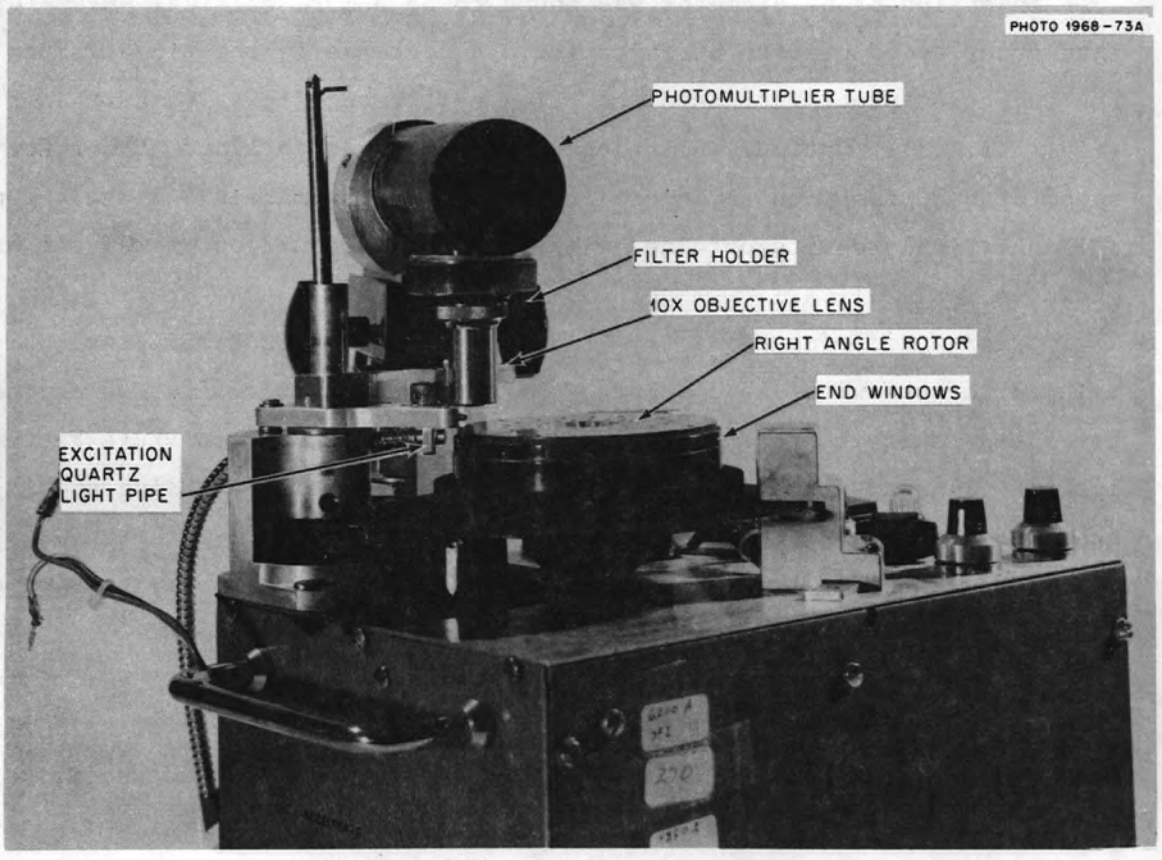

Fig. III.A.3. Optical Configuration of a Monitor Developed to Measure Fluorescence and Light Scattering with a Miniature Fast Analyzer.

Table III.A.1. Comparison of Analytical Results Obtained with Either Discrete[a] or Dynamic[b] Loading of Reagents

Sample No.	Enzyme Activity (I.U. liter^{-1} min^{-1}, 30°C)					
	ALP[c]		LDHL[d]		SGOT[e]	
	Discrete	Dynamic	Discrete	Dynamic	Discrete	Dynamic
1	30.6	31.9	62.8	59.9	11.4	10.8
2	210.2	201.5	217.6	213.2	43.4	41.9
3	37.6	38.9	70.8	67.8	5.7	5.5
4	38.9	39.8	67.9	63.3	6.8	5.2
5	32.5	33.1	62.2	58.9	10.1	8.0
6	58.7	57.7	70.1	76.5	12.9	12.0
7	27.7	27.0	100.0	86.8	11.0	9.6
8	43.9	43.7	97.2	95.9	10.2	8.3
9	42.3	41.6	87.8	87.3	25.0	23.5
10	52.3	52.0	84.4	83.0	14.2	12.6
11	58.7	58.5	93.4	96.8	12.8	8.3
12	56.0	56.9	56.8	53.9	6.7	5.6
13	38.7	38.4	66.6	66.1	6.7	4.0
14	37.2	38.3	68.0	67.5	4.6	3.8
15	30.0	30.5	61.3	57.7	10.3	8.9
16	210.1	202.9	216.2	220.7	42.2	42.3
Average	62.8	62.1	92.7	91.0	14.6	13.1

[a]10 μl sample + 50 μl diluent; 20 μl reagent + 50 μl diluent.

[b]10 μl sample + 50 μl diluent; 60 μl reagent (diluted 1:3).

[c]Conditions: 400 nm; 30°C; dynamic loading speed = 3000 rpm; delay interval = 10 sec; observation interval = 10 sec; number of observations = 20.

[d]Conditions: 340 nm; 30°C; other conditions identical to those in footnote *c*.

[e]Conditions: 340 nm; 30°C; other conditions identical to those in footnote *c*, except observation interval = 15 sec.

system. A semiquantitative evaluation of the rotor and its fluorescence detection sensitivity made using solutions of sodium fluorescein in 0.01 *N* NaOH indicated a detection limit in the range of 1.0×10^{-10} mole/liter. At this level, adsorption of the fluorescence material onto walls of the dilution vials becomes a limiting factor along with blank emission due to many factors, including Raman emission and emission filter leakage of scattered light.

In addition to fluorescence monitoring, the right-angle system can also be used as a light-scattering monitor. In this context one of its obvious applications is in the area of specific protein analyses using agglutination. For example, serum IgG, IgA, IgM, α-1-trypsin inhibitor, transferrin, and C'3 complement factor are among some 40 specific serum proteins that can be assayed. These analyses are based on the aggregation of immunoglobulins with their respective antibodies. This aggregation can be measured by determining the change in light scattering either during the aggregate formation (kinetic) or after aggregate formation (equilibrium).

In an initial study 100 μl of antihuman IgG (purchased from Meloy Laboratories, Inc.), diluted 1/25 with 0.9% NaCl, was pipetted in reagent cavities 1 through 17 of the right-angle rotor. Fifty microliters of diluent was added to sample cavities 1 and 2; 50 μl of human IgG (2.73 mg/ml), diluted 1/1000 in 0.9% NaCl, was added to sample cavities 3 through 6; 50 μl of human IgG (6.1 mg/ml), diluted 1/1000, was added to sample cavities 7 through 10; 50 μl of human IgG (11.5 mg/ml), diluted 1/1000, was added to sample cavities 11 through 14; and samples containing 23.0 mg/ml of human IgG, diluted 1/1000, were added to sample cavities 15 through 17. Excitation was set at 410 nm; the rotor containing the IgG samples and antibody was accelerated to 500 rpm; and the light-scattering intensity was measured initially at 16 min. The results indicated increasing intensity with increasing concentrations of IgG. The system is apparently very sensitive in that it can detect microgram-per-milliliter quantities of IgG and it requires the use of only 4 μl of antibody per test (100 μl of a 1/25 dilution).

The on-line computer permitted a somewhat unique approach to be taken in IgG analysis in that the rate of IgG:anti-IgG aggregation was monitored

with time (Fig. III.A.4). Thus the direct kinetic determination of IgG concentration in a few minutes can be considered. The time required for equilibrium light-scatter procedures is 20 min for IgG and longer for other assays, while a time of 18 hr is generally used for double immunodiffusion procedures which are more commonly employed.

A surface fluorescence optical system (shown in Fig. III.A.5) has been developed for the miniature analyzer. The primary component of the system is a mirror assembly that fits in a slot cut into the end of the filter wedge. The mirror is set at an angle of 30° to the filter holder plane, which results in the excitation light being at an angle of incidence of 60° into the bottom window of the rotating rotor. The mirror extends into the first cylindrical filter holder to permit direction of the exciting light to the maximum possible depth in the cuvet as the cuvet is centered above the aperture of the photomultiplier tube. An objective lens is used to focus the emission signal, and 0.5-in.-diam barrier filters of the desired wavelength are used to isolate the emission signal. A quartz fiber optical bundle had been employed to this point for direction of excitation light to the spinning rotor from a xenon lamp source. The current sensitivity of the system is about 10 ng/ml or 2.5×10^{-8} mole/liter of sodium fluorescein in 0.01 *N* NaOH. The sensitivity of the analyzer can be improved by removing the current 1/4-in. aperture in the photomultiplier holder and replacing it with a variable aperture which would allow most of the emission signal to fall on the photomultiplier tube window in fluorescence mode but would provide a 1/8-in. aperture to be positioned for the photometric mode.

III.A.2. Fluorescence Fast Analyzer

An optical system for measuring fluorescence had been developed for use with the Fast Analyzer.[3,4] The objective of this development was to obtain an instrument that could be used either as a multicuvet spectrophotometer or fluorometer through a single interconversion of the optical system. This goal was achieved, and fluorometric chemistries are now being developed for use with the system.

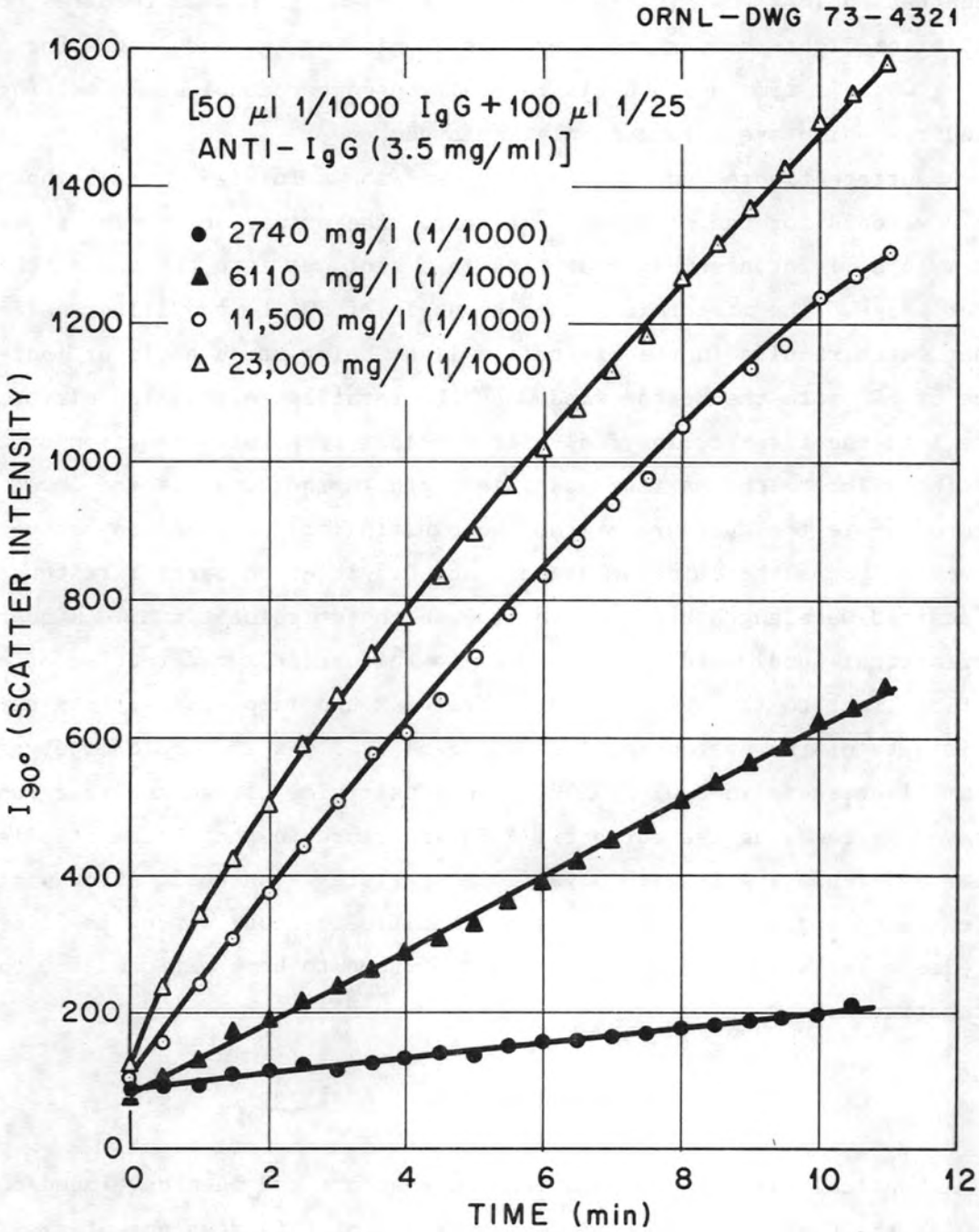

Fig. III.A.4. The Interaction of Human IgG:Anti-IgG vs Time and as a Function of IgG Concentration.

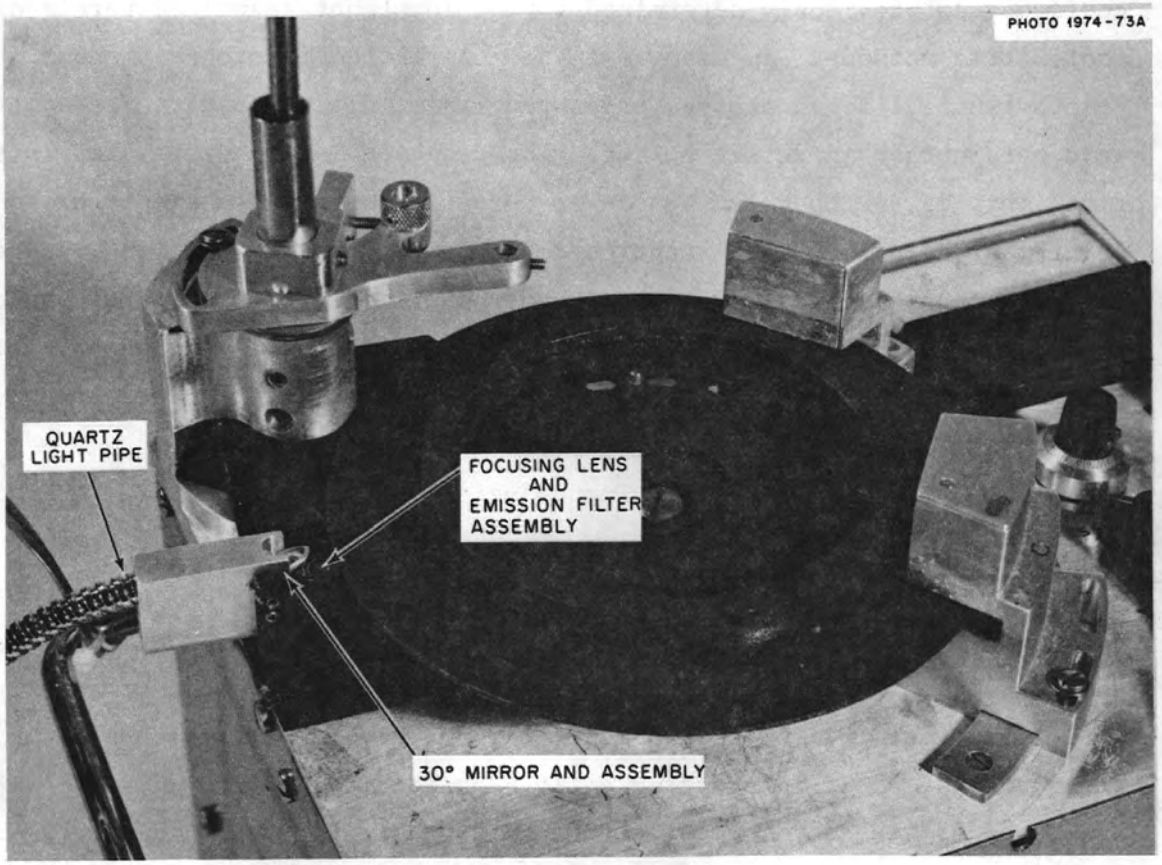

Fig. III.A.5. Fluorometric Monitor Used to Measure Surface Fluorescence with a Miniature Fast Analyzer.

III.A.2.a. Enzyme Analyses

The advantages of fluorometric substrate and enzyme activity assays are sensitivity (e.g., they are 100 to 1000 times more sensitive than photometric measurements) and the availability of a number of fluorogenic compounds which permit direct equilibrium or kinetic assay of substances which could not be previously determined (or at least not readily determined) by photometric methods. An example is the use of the fluorogenic substrate 4-methyl-umbelliferyl phosphate for the rapid, direct, kinetic assay of acid phosphatase in acidic buffer. The reaction is immediate upon initiation, that is, without lag phases or necessity of pH adjustment for endpoint monitoring, and the reaction does not involve the problems inherent in diazo coupling (to produce colored products) that is currently employed in direct, kinetic spectrophotometric assays. By changing to an alkaline pH, the method can also be used to determine alkaline phosphatase activity.

III.A.2.b. Immunological Assays

The development and adaptation of immunological assays to the Fast Analyzer (miniature and 15-place) is being approached in three ways: (1) the development of light-scattering monitors for measurement of particulate formation; (2) the development of fluorescence polarization techniques for quantitation of interacting antigen-antibody or hapten-antibody systems, and (3) the development of competitive binding fluoroimmunoassays.

Light Scattering. -- The progress in development of immunological light-scatter measurement techniques for the Fast Analyzer has been discussed previously in Sect. III.A.1.b. It should be noted that the use of the light-scattering technique for measurement of serum protein provides selectivity and the sensitivity needed to measure serum proteins at concentrations that were previously difficult to measure, i.e., μg/ml to mg/ml of individual protein.

Fluorescence Polarization. -- Fluorescence polarization, although somewhat limited in sensitivity, offers one method for the direct quantitation of equilibrated solutions of antigen-antibody or hapten-antibody

without first separating the bound antigen-antibody complex from free antigen. This is particularly promising in the area of quantitation of circulating antibody formed in autoimmune disease or the infection of the body by foreign material. It has been demonstrated that fluorescence polarization measurements can be made by using the 15-place Fluorometric Fast Analyzer.[4] This appears to be an open area for development to produce an automated fluorescence polarization immunological assay for circulating antibody and will be investigated further.

Competitive Binding Assays. -- The equilibration of an antigen with its specific antibody, or the equilibration of a substrate with a specific binding protein and then the subsequent equilibration of the same system with a tracer-labeled antigen or solute, produces a means of quantitation of the original concentration of the antigen or substrate by measuring the amount of tracer bound or the amount of free tracer remaining in solution after removing the bound complex. This is represented by the following equation:

$$\text{Antigen} + \text{Antibody} \rightleftharpoons \text{Antigen-Antibody Complex}$$

$$\text{Labeled Antigen*} + \text{Antibody} \rightleftharpoons \text{Antigen*-Antibody Complex}$$

The amount of labeled antigen bound to antibody is an inverse function of the amount of antigen present in the system. The same consideration applies to hapten-antibody and specific protein-binding systems.

Radioactive labeled compounds are most commonly used as tracers, and they have provided the sensitivity required for measuring substances in serum in the concentration range of 10^{-11} to 10^{-12} *M*. If a reasonably stable fluorescence labeled compound can be obtained, if the sensitivity required for these assays can be achieved, and if much of the tedium can be eliminated from these assays by automation of separation steps and rapid measurement of remaining fluorescence tracer, then the fluoroimmunoassay should provide many advantages over the radioimmune methods.

Insulin has been labeled with fluorescein isothiocyanate (FITC), and we plan to use this product in the development of a fluoroimmunoassay for insulin. In addition, we have begun the investigation of the possible development of a fluoroimmunoassay for L-thyroxine through the use of fluorescamine-labeled L-thyroxine.

III.A.3. Evaluation and Operation of a Fast Analyzer in a Clinical Laboratory

A 15-place GeMSAEC Fast Analyzer has been routinely operated in the clinical laboratory of the Health Division of the Oak Ridge National Labratory for the past two years. This routine operation has allowed an extremely useful evaluation to be made at the same time that it provided useful medical information for the ORNL employees. For example, Laboratory personnel who undergo a periodic health examination are asked to present themselves at the time of the examination in a fasting state. The following serum tests are then performed with the use of the Fast Analyzer: glucose (fasting and 2-hr post-100-g glucose load), triglycerides (fasting), SGOT, LDH, and alkaline phosphatase.

Typical of the data obtained with the routine use of the Fast Analyzer is that obtained with the glucose and triglyceride analyses. Table III.A.2 is a compilation of the results for serum glucose analyses from the ORNL population by sex and age. Such statistical results provide several benefits. First, the normal range of serum glucose as a function of age and sex is established for both fasting and 2-hr post glucose loading. Second, it is shown that there is a definite age dependence in the data and this is particularly evident after the glucose loading. Third, the fasting serum glucose value and standard deviations are a function of age, but, the serum analysis for glucose 2 hr after a glucose load of 100 g reflects the diverse response to this stress, since in some age groups the standard deviation doubles. This demonstrates a reduction in the ability to control glucose with an increase in age and can be viewed as metabolic data reflecting the aging process and loss of glucose control as one facet of human aging.

During the past several months, fasting serum triglyceride values have been routinely obtained as a part of the periodic health examination of ORNL employees. These triglyceride analyses were performed using an enzymic procedure. The statistical data for 543 analyses are shown in Table III.A.3. The data are broken down with respect to age and sex. No attempt was made to eliminate the several samples within this group that

Table III.A.2. Serum Glucose Data from the ORNL Population

Population Description	Number of Subjects	Serum Glucose Concentration After Fasting		Serum Glucose Concentration 2 hr After a 100-g Glucose Loading	
		Mean Level (mg/100 ml)	Range[a] (mg/100 ml)	Mean Value	Range[a]
Total population	605	87.5	68 - 107		39 - 152
Total male	481	89	70 - 106	87	40 - 155
20-30 years	18	88	74 - 101	96	59 - 122
30-40 years	102	86	69 - 103	86	42 - 131
40-50 years	205	88	72 - 105	86	46 - 148
50-60 years	135	90	71 - 110	104	37 - 171
>60 years	21	93	65 - 121	120	42 - 193
Total female	124	83	62 - 105	88	40 - 137
20-30 years	28	77	60 - 95	85	51 - 125
30-40 years	20	80	67 - 93	89	33 - 137
40-50 years	47	86	62 - 110	94	43 - 136
50-60 years	24	88	68 - 108	85	35 - 153
>60 years	5	85	77 - 93	84	61 - 108

[a]Mean value ± two standard deviations.

Table III.A.3. Serum Triglyceride Concentrations of ORNL Employees

Population Range (Years)	Number of Samples	Mean Concentration (mg/100 ml)	Concentration Range[a]
Male			
All ages	460	142	64 - 220
20-29	22	138	71 - 205
30-39	115	124	65 - 185
40-49	171	152	66 - 283
50-59	133	143	68 - 218
60-65	19	178	75 - 220
Female			
All ages	83	105	55 - 160
20-29	25	95	72 - 118
30-39	17	96	61 - 127
40-49	22	94	50 - 138
50-59	18	141	67 - 215
Total population			
All ages	543	137	60 - 213

[a]Mean value ± two standard deviations.

were highly elevated (e.g., above 400 mg/100 ml as triolein) as has been done in other similar studies. The greater number in this population study are in the age group of 35 to 50 years of age. The trend is towards increasing values with age and lower values for females (Table III.A.3). A histogram of the serum triglyceride levels in the ORNL male population from age 30 to 60 is shown broken down in three groups in Fig. III.A.6. Serum triglyceride values for the age group of 30 to 39 years (123 samples) represents a normal pattern with 86% of the population below 185 mg/100 ml and 95% below 230 mg/100 ml. In the population range of 40 to 49 years (group *b*, N = 171) triglyceride values increase, with several values greater than 400 mg/100 ml. Although 81% of the population is below 185 mg/100 ml, the range has increased, and 95% of the population is below 280 mg/100 ml. The last age group, 50 to 59 years, again displays a much broader distribution with 80% below 185 mg/100 ml but 95% below 280 mg/100 ml.

This type of data demonstrates the functioning role of the Fast Analyzer in the ORNL Health Division chemistry laboratory. In addition, such information obtained from a large static population that represents a variety of occupations can be used to study preclinical disease in an apparently healthy population. This is not possible with hospital populations. This is not to suggest the use of the population for experimentation, but to suggest the effective use of existing population data to obtain more extensive information concerning the relationship between test results and subclinical disease. This is only possible when reliable, accurate, and precise analytical instrumentation is available.

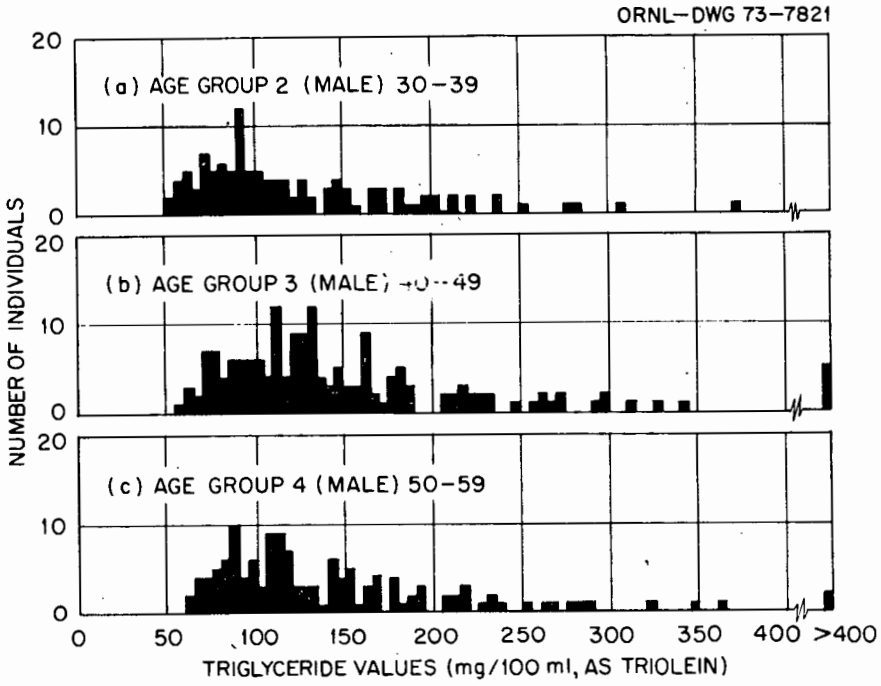

Fig. III.A.6. Histogram of Serum Triglyceride Values for Three Age Groups of Male ORNL Employees.

III.B. HIGH-RESOLUTION ANALYTICAL SYSTEMS

J. E. Mrochek, D. D. Chilcote, S. R. Dinsmore, G. Jones,
S. Katz, N. E. Lee, W. W. Pitt, and C. D. Scott

A number of disease states may give early warning of their onset by subtle changes in excretion levels for molecular constituents in physiological body fluids. Detection and monitoring of such biochemical indicators will require that research and clinical laboratories have automated, high-resolution analytical systems with which to identify and measure large numbers of the molecular constituents. To attempt to fill this need, a number of liquid chromatographic systems are being developed.

III.B.1. Prototype Development

Prototype UV Analyzers are currently undergoing field evaluation at the Clinical Center of the National Institutes of Health, Duke University Medical Center, and the Texas Medical Center at Houston. Three computer systems for on-line data analyses for these analyzers have been constructed and are being installed. Two new UV Analyzer prototype systems are being fabricated for other government agencies.

III.B.1.a. Prototype Evaluation

All prototype systems in the field, with the exception of the UV Analyzer at Duke University, have exhibited reliable, trouble-free operation during the current report period. The Duke machine has been plagued by repeated pump failures, and a replacement pump has recently been installed.

III.B.1.b. Fabrication of Computer Systems

Three miniaturized, computer-based data acquisition and analyses systems have been developed and are being installed for on-line, real-time analysis of the data from a UV Analyzer or any comparable chromatographic

system. The computer systems are equipped with 12K words of fast memory, a dual cassette tape transport, a real-time clock, an automatic program loader, a digital input/output interface, and an analog input subsystem (ADC).[1] These interfaces enable the computer to simultaneously collect data from two chromatographs while analyzing stored data from one chromatographic analysis.

The first computer system was delivered to the Clinical Center of NIH on June 26, 1973. This system is presently interfaced to two UV Analyzers. Although data acquisition from the two systems is often simultaneous, the analysis of the data is always sequential. The second computer system was delivered to Duke University on August 28, 1973. Late October is the tentative date set for delivery of the third computer system to the University of Texas Medical Center at Houston. The last two systems will initially be interfaced to a single UV Analyzer.

III.B.1.c. Fabrication of UV Prototypes

Construction of two prototype UV Analyzers for delivery to other government agencies was initiated during this report period. A Mark II-A UV Analyzer is being fabricated for the Environmental Protection Agency to be used at the Southeast Environmental Research Laboratory in Athens, Georgia. A Mark II-A UV Analyzer, modified for rapid analysis for nucleosides (see Sect. III.B.2.b), is being fabricated for the National Cancer Institute.

III.B.2. Systems Development

Development efforts are continuing on high-resolution separation systems and more sensitive detection devices for application in biomedical research and the clinical laboratory. As new developments occur in these areas, their applications to practical problems in the separation and detection of constituents present in physiologic body fluids are evaluated.

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[1] *Molecular Anatomy (MAN) Program Semiann. Progr. Rept., Sept. 1, 1972, to Feb. 28, 1973*, ORNL-4867.

III.B.2.a. Cation Exchange Separation of Ninhydrin-Positive Constituents in Urine and Blood Sera

The compact, high-resolution, ninhydrin-positive compound analyzer shown in Fig. III.B.1 has been developed for use as a high-resolution system for separation and detection of amino acids found in urine and blood sera. Two ion-exchange columns have been used on the analyzer: (1) a 0.45-by-200-cm column for the urine analysis, and (2) a 0.22-by-300-cm column for the analysis of blood sera. The columns are fabricated from Type 316 stainless steel tubing and are packed with Aminex A-6 cation-exchange resin with a particle size of 15 to 19 μ. The results of the separation of a typical urine sample are shown in Fig. III.B.2.

III.B.2.b. Analysis of Nucleosides and N-Bases

The nucleoside and N-base analysis system, developed to the point described in a previous report,[1] was unable to separate 7-methylguanine and N^2-dimethylguanosine. These compounds are of interest since both are present in human urine, and the level of N^2-dimethylguanosine is suspected to be elevated in the urine of cancer patients.[2] Recent studies have demonstrated that these compounds may be separated by decreasing the pH of the eluent from 4.2 to 3.7. The elution volumes of various nucleosides and N-bases as a function of pH and temperature are listed in Table III.B.1.

III.B.2.c. Analysis of Blood Serum

Application of the UV Analyzer to the analysis of blood serum has not been significantly pursued since the uv response of only a few constituents permits satisfactory detection at their prevailing concentrations. The

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[2] Waalkes, T. P., S. R. Dinsmore, and J. E. Mrochek. "Increased Urinary Excretion of the Nucleosides, N^2,N^2-Dimethylguanosine, 1-Methylinosine, and Pseudouridine by Cancer Patients." *J. Natl. Cancer Inst.* *51*, 271 (1973).

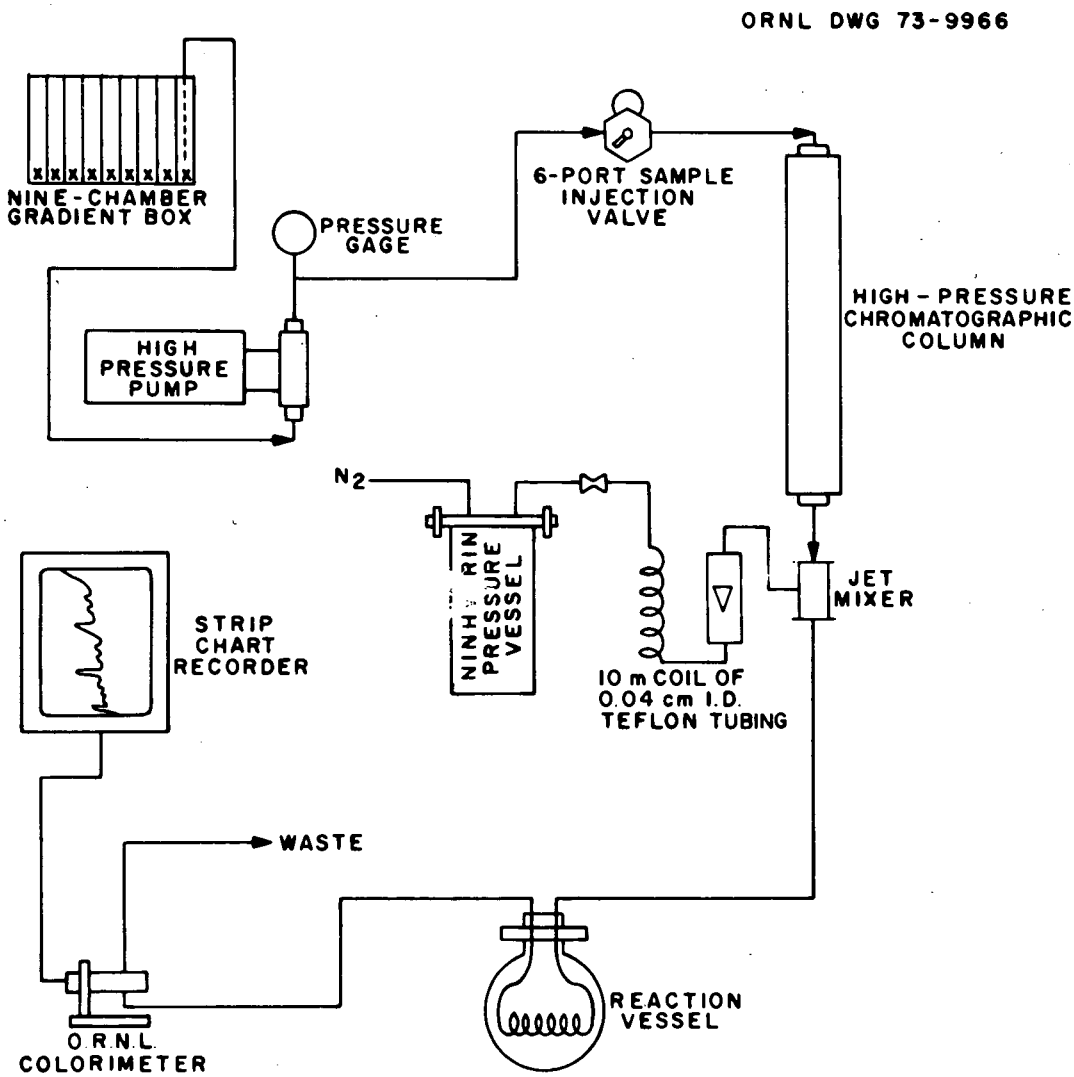

Fig. III.B.1. Analyzer for Ninhydrin-Positive Compounds in Physiologic Fluids.

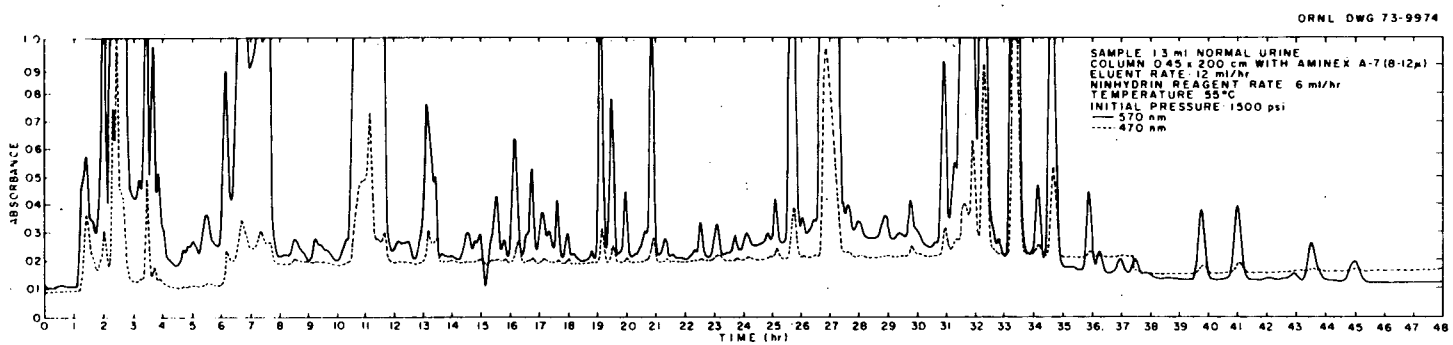

Fig. III.B.2. Chromatogram of Ninhydrin-Positive Compounds Separated from a Composite Normal Urine.

Table III.B.1. Elution Volume of Nucleosides and N-Bases as a Function of pH and Temperature Profile

Compound	Elution Volume at Given Parameters					
pH:	3.70		4.20		4.40	
Temperature Controller Set:	1.61	2.70	1.61	2.70	1.61	2.70
Cytidine	18.6	18.4	20.5	19.4	22.7	21.3
Uracil	30.7	31.0	31.4	30.8	31.8	31.7
Uridine	36.3	36.8	37.1	36.6	37.5	37.6
1-Methylinosine	42.5	43.6	43.2	43.7	43.9	45.7
7-Methylxanthine	47.1	48.0	49.1	48.7	50.3	50.6
Hypoxanthine	51.7	53.0	52.6	53.4	53.3	55.0
Inosine	54.4	57.5	54.9	57.9	55.8	60.1
1-Methylguanosine	59.3	62.5	59.7	62.7	60.1	64.6
7-Methylguanine	63.0	66.5	67.0	72.0	67.7	76.1
N^2-Dimethylguanosine	66.2	71.7	67.0	72.0	67.7	75
3-Methylxanthine	73.2	76.8	75.5	78.5	73.2	83.4
N^2,7-Dimethylguanine	80.7	85.6	89.0	94.2		
Xanthine		82.7			81.1	97.0

application of the cerate oxidative detector[3] in series with the uv detector provides additional, sensitive detection capability to a liquid chromatography system for analysis of blood serum. The chromatographic system and its operation are similar to that described earlier for urine analysis.[3] The volume of sample in the sample loop has been increased from 0.27 ml for urine samples to 0.60 ml for ultrafiltered blood serum; larger volumes appear to introduce peak broadening, especially for early-eluting compounds. Several interrelated parameters were changed to permit the total elution to be completed in less than 20 hr with minimal effect upon the separation. Those changes are: the column length was decreased from 150 to 100 cm; the eluent was reduced in total volume from 260 to 225 cc and prepared in a 9-chamber gradient box with 25 g of acetate buffer solution (pH 4) in each chamber (0.015 *M* in 1 and 2, 4 *M* in 3 and 4, and 6.0 *M* in the remaining 5 chambers); and the column temperature was increased to 60°C at 1.3 hr and was held there for the remainder of the analysis.

As an example of application of the system, chromatograms were obtained from samples of a clinically normal individual (1) after overnight fasting, (2) 1/2 hr after ingesting a glucose dose, (3) 1 hr after ingesting the glucose dose, and (4) 3 hr after ingesting the glucose dose. The chromatograms, shown in Fig. III.B.3, demonstrate the unique ability of this type of analytical system to measure the effect of a single parameter upon many blood constituents. Over 30 uv chromatographic peaks and 30 cerate oxidative detection (COD) peaks were detected in each of the chromatograms, representing more than 50 constituents.

Hippuric acid and several other aromatic acids were tentatively identified by their elution positions; these have not heretofore been reported in blood. The levels of detection, in the order of 0.01 to 0.1 μg/cc of blood, should be useful in defining the absence of such important compounds as 5-hydroxyindoleacetic acid. The larger peaks have been verified

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[3]Katz, S., W. W. Pitt, Jr., and G. Jones, Jr. "Sensitive Fluorescence Monitoring of Aromatic Acids After Anion Exchange Chromatography of Body Fluids." *Clin. Chem. 19*, 817 (1973).

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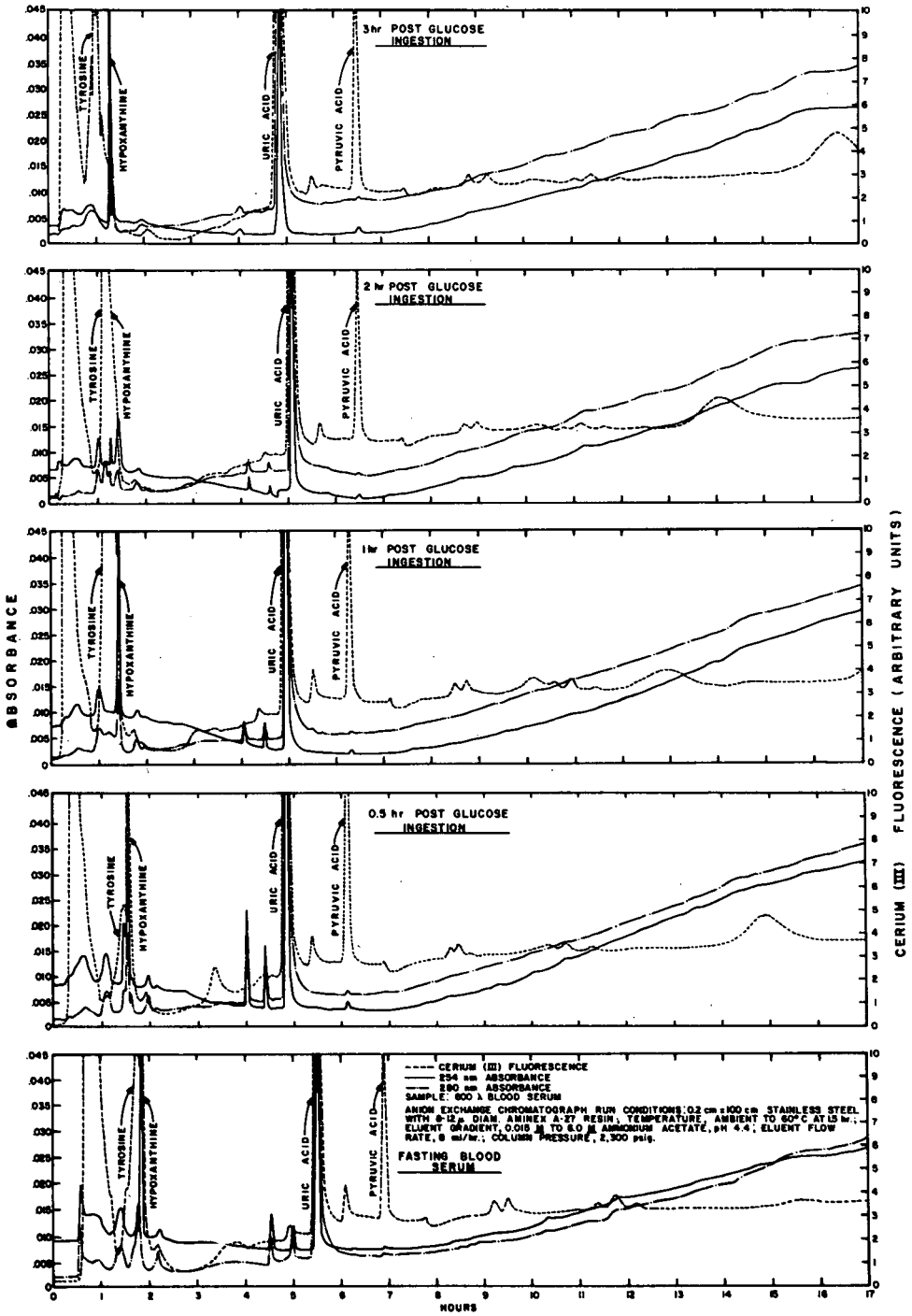

Fig. III.B.3. Chromatogram of Blood Serum on the UV Analyzer with the Cerate Oxidimetric Detector.

as pseudouridine, tyrosine, hypoxanthine, uric acid, and pyruvic acid by collection of eluate fractions followed by rechromatography on a cation-exchange column. Smaller peaks coincide, in a number of cases, with the elution position and relative responses of known aliphatic and aromatic acids. A large sample of 500 cc of pooled blood serum has been collected and is being processed for concentration and separation on the preparative uv system for identification studies.

III.B.2.d. The Application of Fluorescamine to the Monitoring of Urinary Polyamines

Studies have continued in an effort to adapt the reagent known as fluorescamine (Fluram™)[4] to a sensitive analysis for polyamines in physiologic body fluids. In previously reported studies[1] a useful ion-exchange separation of these compounds was indicated, and efforts have continued to improve this separation and adapt it to a complete, automated system.

Figure III.B.4 is a schematic flow diagram of the ion-exchange chromatograph which was constructed. Three Milton-Roy minipumps served to pump the column eluent, the buffering agent (0.1 *M* H_3BO_3), and the fluorescamine solution through the system. Four chambers of a gradient box supplied the eluent, which was pumped through a six-port sample-injection valve supplied with a 0.450-ml sample loop. A jacketed nickel column (0.45 cm ID by 15 cm) was used in this investigation. Nickel was used in order to minimize the possible corrosive effects of sodium chloride solutions. The column was slurry packed with Aminex A-5 resin (13 to 20 μ) which was supported by a stainless steel frit. The column was operated at 70°C throughout the analysis.

In order to form the fluorescamine derivatives of the polyamines at the optimum pH for fluorescence measurement, the pH of the column effluent was readjusted by mixing it with 0.1 *M* boric acid solution in an annular mixer as it left the column. The buffered stream was then fed to a T-mixer

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[4]Udenfriend, S., S. Stein, P. Bohlen, W. Dairman, W. Leimgruber, and M. Weigele. "Fluorescamine: A Reagent for Assay of Amino Acids, Proteins, and Primary Amines in the Picomole Range." *Science 178*, 871 (1972).

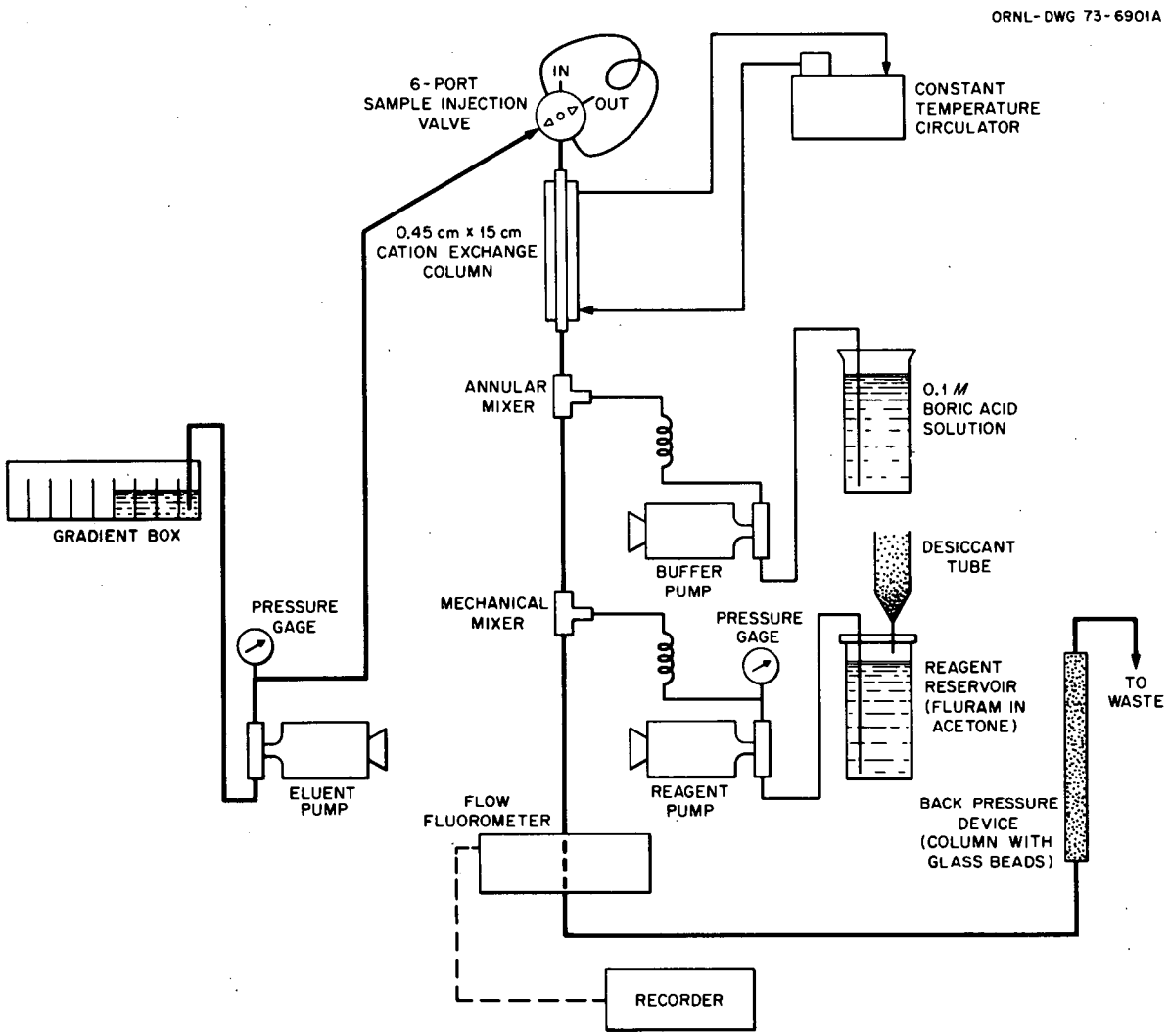

Fig. III.B.4. Polyamine Analyzer Using a Cation Exchange Separation with Fluorescence Monitor.

supplied with a small magnetic stirring bar, where the fluorescamine reagent stream was introduced. A fluorescence flow monitor downstream of the mixer was used to detect components eluting from the column.

A typical separation of the five most important polyamines requires 2 hr. Typical chromatographic analyses of the urine from a clinically normal subject and that from a cancer patient are shown in Fig. III.B.5. Figure III.B.5*a* represents the chromatogram of a hydrolyzed, pooled urine sample obtained from eight normal males. Peaks 1, 3, 4, and 5 appear in the normal retention positions for 1,3-diaminopropane, putrescine, spermidine, and cadaverine respectively. A smaller unknown peak (No. 2) also appears. The chromatogram in Fig. III.B.5*b* represents a hydrolyzed urine sample from a cancer patient. Peaks 6 and 7 represent components located in the normal retention positions for putrescine and spermidine. Based on the established calibrations, the normal urine sample contains 0.7 μg of putrescine/ml, 1.5 μg of spermidine/ml, and a trace of cadaverine. The chromatogram in Fig. III.B.5*b* shows the presence of over 2.0 μg of putrescine/ml and 4.0 μg of spermidine/ml. It can be seen in these urine analyses that the amino acids and other less basic components elute early under the chromatographic conditions utilized, leaving a reasonably clear area for the elution of the polyamines.

III.B.2.e. Multicomponent Separation by Affinity Chromatography

A preliminary study was performed on the feasibility of utilizing affinity chromatography to achieve multicomponent separation. The studies were carried out with an agarose support to which the antibodies of either albumin or IgG were attached. A peristaltic pump was used to force eluent through a sample injection valve and a 15-mm-ID column containing the sorbent. The column eluent was monitored at 280 nm by a uv photometer.

A phosphate buffer (pH 7.0) was selected as the washing buffer and 2 *M* sodium perchlorate was used as the eluting buffer, since these were found to be compatible with the two protein-antibody pairs selected for testing. The tests showed that an analytical chromatographic peak could be obtained in as little as 45 min with the analytical peak eluting immediately after the change of buffer.

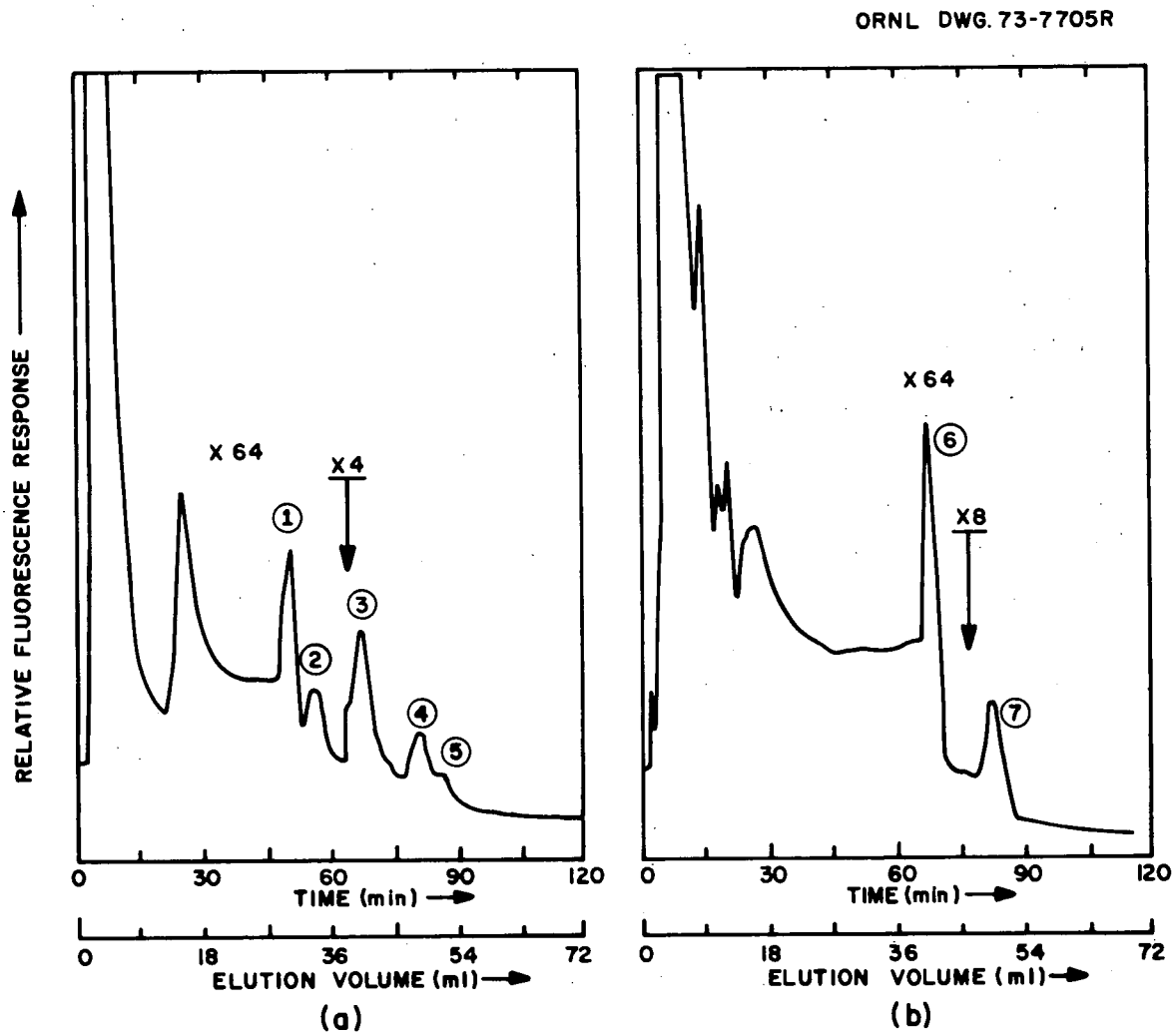

Fig. III.B.5. Chromatographic Separation of Urinary Polyamines with Fluorescamine Monitoring. (*a*) Chromatogram of Polyamines in "Normal" Urine. (*b*) Chromatogram of Polyamines in Urine of a Cancer Patient.

A new design configuration whereby multiple analyses could be performed was tested. This design involved close coupling of affinity and centrifugal chromatography. It included a rotor containing a number of columns, each with a different antibody coupled to its support. A single flow system and detector was used to perform multiple protein separations from a single sample. As described previously,[1] centrifugal force was used to force the eluent through the columns, with the effluent from each column being monitored by a single, stationary photometer. A single-tube mockup of this design was tested and showed that satisfactory physical characteristics, i.e., adequate flow through a column of sufficient capacity for detection, were achieved to demonstrate feasibility of the design.

III.B.2.f. Preparation of Pellicular Gel Filtration Media

Gel filtration presents an attractive approach to the separation of large macromolecules. The notable success of pellicular resins in ion-exchange and adsorption chromatography suggests this approach be considered for gel filtration as well. A solid core would provide mechanical stability, and a thin film of gel would minimize adverse mass transfer effects. The problem is how to apply the gel layer to a solid core. One approach is to form a slurry of the liquified gel and the solid core particles, then nebulize this slurry to disperse the particulates (now coated with a thin film of gel) into an environment conducive to rapid gellation. The feasibility of nebulizing a slurry of solid particles of approximately 0.1 to 1.0 μ diameter has already been demonstrated in an entirely different application.[5]

Preliminary investigations indicate that this approach will be useful. We successfully nebulized slurries of hot agarose gel and small glass spheres (44 to 57 μ), after which the atomized slurry was collected

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[5] Israel, G. W., and S. K. Friedlander. "High Speed Beams of Small Particles." *J. Colloid and Interfacial Sci.* *24*, 330 (1967).

in an ice-water bath to promote rapid gellation. Some large gel plaques were also formed on the surface of the cold-water bath; this will necessitate additional development work to arrive at the proper operating conditions. In the future, smaller glass spheres will be used to avoid plugging the nebulizer orifice while allowing a sufficiently high linear velocity of the slurry.

III.B.3. Identification of Body Fluid Constituents

Eight new uv-absorbing compounds have been identified, increasing the total number of identified constituents to 127 (including carbohydrates, the total is 139). The new identifications are listed in Table III.B.2, together with origins of the urine samples from which they were isolated. A ninth compound, listed in Table III.B.2 as a metabolite of 6-thioguanine, was previously tentatively identified as having the methyl substituted on the free amino group.[6] Mass spectra data from a number of methyl-substituted thioguanines have conclusively shown that the metabolite isolated from leukemic patients receiving 6-thioguanine is the riboside, methylated on the sulfur group.

III.B.4. Experimental Results and Applications

The chromatographic systems described in the previous sections have been applied to the analysis of body fluids obtained from patients having various pathologic conditions.

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[6] *Molecular Anatomy (MAN) Program 1st Semiann. Progr. Rept., Mar. 1 to Aug. 31, 1972*, ORNL-4815.

Table III.B.2. Identification of Urinary Metabolites During the Past Six Months

Compound	Sample Origin
β-(3-Methoxy-4-hydroxyphenyl)hydracrylic acid	Normal and pathologic samples
α-Methoxy-α-(3-methoxy-4-hydroxyphenyl)acetic acid	Lesch-Nyhan syndrome, mental retardation, a number of different types of cancer
4-Hydroxyacetanilide (sulfate conjugate)	Drug metabolite
3-Methoxy-4-hydroxyacetanilide (sulfate conjugate)	Drug metabolite
1,3-Dimethyluric acid	Normal and pathologic samples, dietary source
3,7-Dimethyluric acid	Normal and pathologic samples, dietary source
1-Methyluric acid	Normal and pathologic samples, dietary source
6-Thiomethylguanosine[a]	Metabolite of 6-thioguanine in leukemia
3-Methoxy-4-hydroxycinnamic acid (glycine conjugate)	Increased in a number of pathologic conditions

[a]Previously identified (tentatively) as N-methyl-6-thioguanosine.

III.B.4.a. Urinary Nucleoside Excretion by Patients with Cancer

Progress continues on the analysis of urine samples[7] from patients with various neoplastic diseases by use of high-resolution analytical systems using high-pressure liquid chromatography. The primary purpose of this effort is to find and identify urinary excretion products which might serve as biochemical indicators of the presence of malignant cell populations. Current studies are concentrated on the measurement of three urinary nucleosides, pseudouridine (Ψ), 1-methylinosine (M_1I), and N^2-dimethylguanosine (M_2^2G), in urine samples from normal subjects and patients with cancer.

In previous studies[1,2,6] the methylated N-bases, 1-methylhypoxanthine and N^2-dimethylguanine, were shown to be excreted primarily in the form of their nucleosides, M_1I and M_2^2G. The only methylated free N-base which we have found to be excreted in substantial amounts is 7-methylguanine.

Previously, urinary excretion levels of Ψ, M_1I, and M_2^2G were reported for a number of normal adults and patients with various malignant diseases.[1] We have now analyzed a total of 76 urine samples from cancer patients for Ψ and 49 samples for M_1I and M_2^2G. Normal excretion levels for adults (ages 15-53) averaged 67.4 mg/24 hr with a standard deviation (S.D.) of 14.3 mg/24 hr for Ψ, 4.0 mg/24 hr (S.D. = 1.0 mg/24 hr) for M_1I, and 3.9 mg/24 hr (S.D. = 1.3 mg/24 hr) for M_2^2G. Similar data for children (ages 3-13) were 35.4 mg/24 hr (S.D. = 8.5 mg/24 hr) for Ψ, 2.5 mg/24 hr (S.D. = 0.9 mg/24 hr) for M_1I, and 2.5 mg/24 hr (S.D. = 1.0 mg/24 hr) for M_2^2G. The data from urine of cancer patients, summarized in Figs. III.B.6 and III.B.7, show that 55% of the urinary excretion from cancer patients for Ψ and 57% of the M_1I and M_2^2G were more than one standard deviation above the results for normal subjects.

Samples from three cancer patients were analyzed for the three nucleosides before and after a chemotherapeutic treatment regimen. The results,

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[7] Samples and funding provided by the National Cancer Institute.

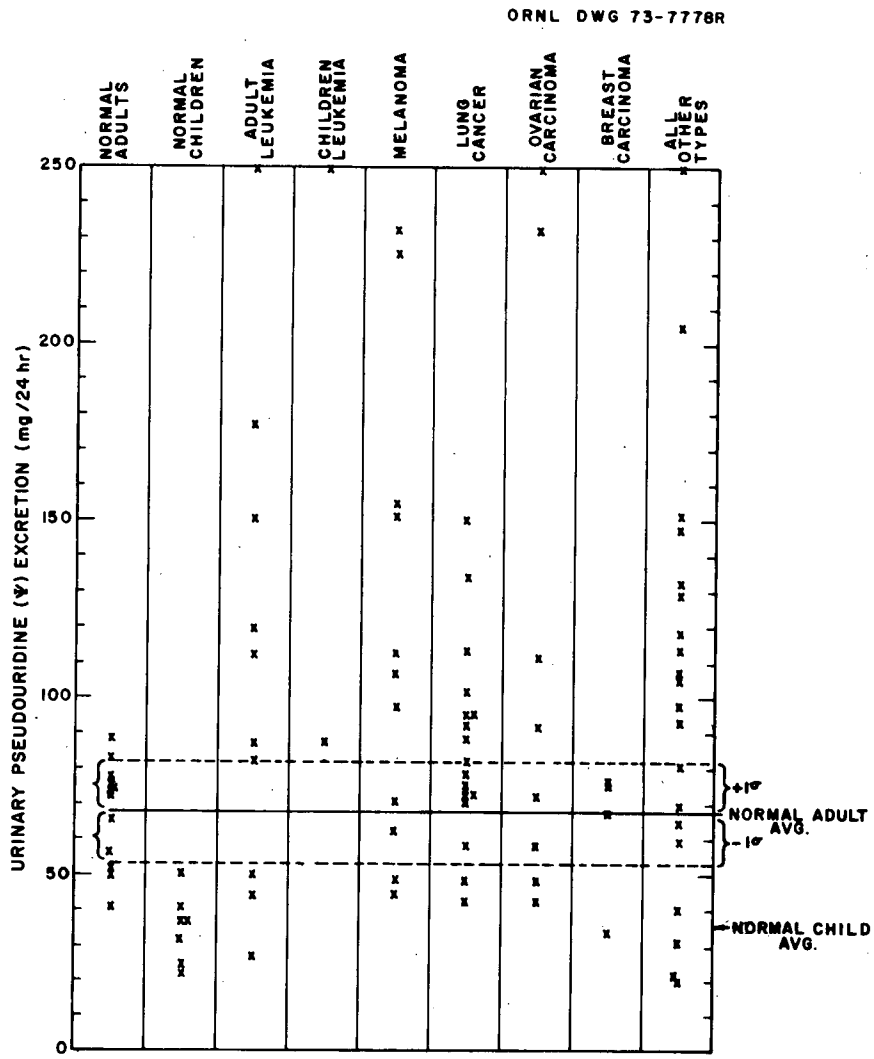

Fig. III.B.6. Comparison of Pseudouridine Excretion of Normal Subjects and Cancer Patients.

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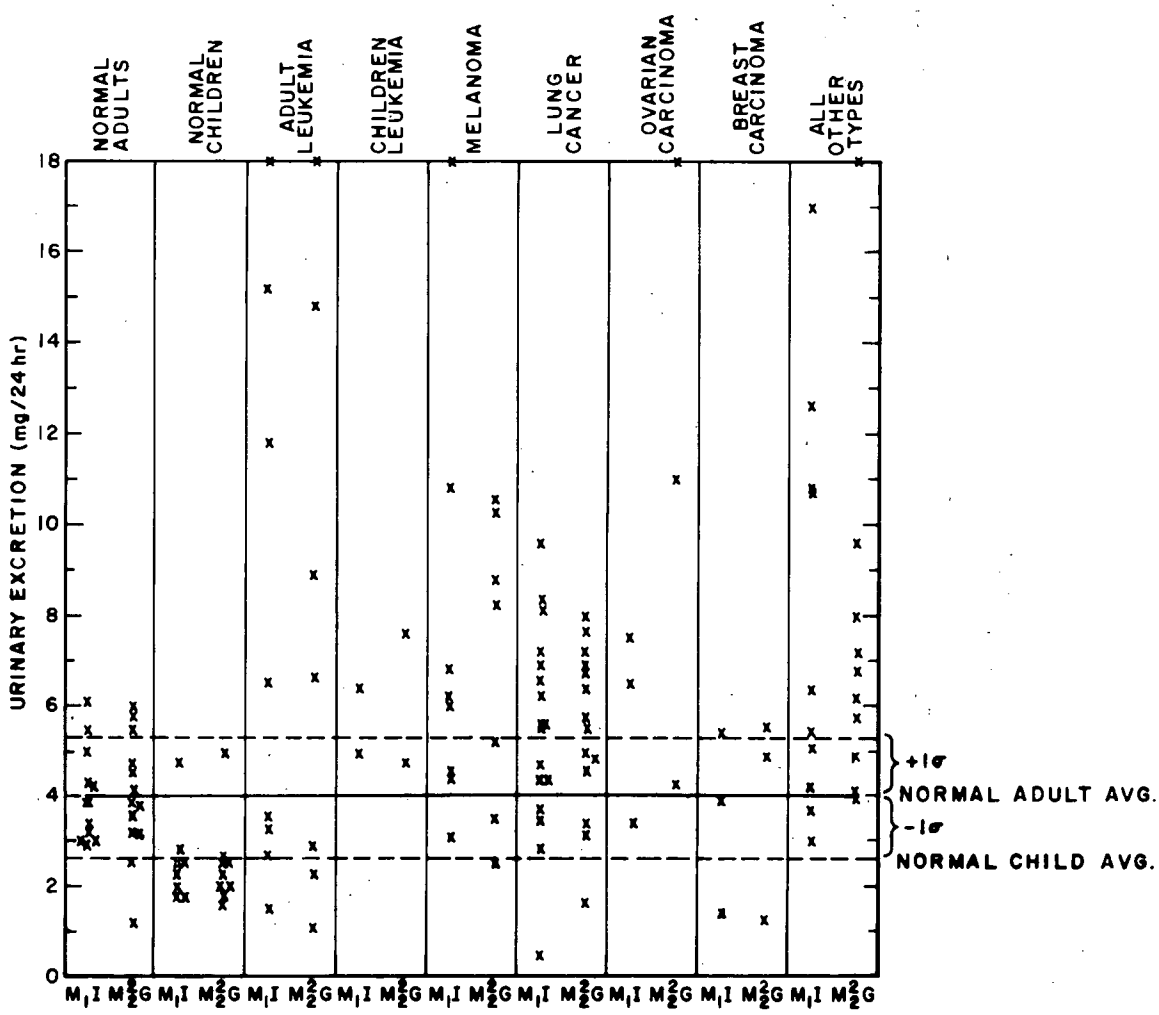

Fig. III.B.7. Comparison of 1-Methylinosine (M_1I) and N^2-Dimethylguanosine (M_2^2G) Excretions of Normal Subjects and Cancer Patients.

shown in Table III.B.3, indicate a reduction in the excretion level of the three nucleosides for the patients with lung and breast cancer; however, an increased excretion was observed for the patient with stomach cancer in the posttreatment samples.

Table III.B.3. Effect of a Chemotherapeutic Treatment Regimen on Urinary Nucleoside Excretion

Sample	Diagnosis	Age, Sex	Treatment	Excretion (mg/24 hr)		
				Ψ	M_1I	M_2^2G
167(010)	Stomach	51, F	No	105.2	6.4	6.8
170(013)	cancer		Yes	122.6	8.3	9.2
127	Lung	68, M	No	113.7	9.6	8.0
157	cancer		Yes	59.0	5.6	7.4
145(003)	Breast	47, F	No	66.9	5.4	5.6
190(026)	cancer		Yes	56.6	1.3	3.6

Urine samples from a 20-year-old male with Burkitt's lymphoma were analyzed during an initial, untreated, active state and during remission when he was undergoing chemotherapy. Urinary nucleoside excretion rates shown in Table III.B.4 indicate a substantial reduction in the excretion of Ψ, M_1I, and M_2^2G when the disease was in remission.

Table III.B.4. Excretion of Nucleosides from One Subject During Active and Remission Phases of Burkitt's Lymphoma

Disease Status	Drugs	Excretion (mg/24 hr)		
		Ψ	M_1I	M_2^2G
Active	None	210	12.6	9.6
Remission	BCG	77	3.3	3.4

The data obtained thus far are interesting, but the possible use of these three urinary nucleosides as biochemical indicators of malignant cell populations remains to be tested. The incidence of elevated excretion in patients with malignant disease seems to be only about 50%, which is not very encouraging. Additional studies are necessary during the course of treatment to relate physical and other signs of the status of the disease to nucleoside excretion.

III.B.4.b. The Effects of L-Dopa and a Dopa Decarboxylase Inhibitor on Metabolite Excretion

The usual therapeutic treatment of Parkinson's disease with L-Dopa involves gradual increases in the dosage of the exogenous drug to relatively large amounts.[8,9] Urine samples from two patients with Parkinson's disease receiving 4 and 4.9 g/24 hr, respectively, of L-Dopa were chromatographed on the UV Analyzer. The chromatographic pattern obtained for patient 1 is shown in Fig. III.B.8, where the shaded peaks represent new constituents or normal constituents which were excreted in larger than normal quantities. Estimated 24-hr excretions for some of the identified compounds are compared in Table III.B.5. The normetanephrine metabolite, VMA, was identified and found to be present in relatively small quantities. Use of the cerate oxidimetric detector may enable accurate quantification of this important metabolite.

In a second series of experiments, urine samples obtained from three patients with Parkinson's disease who were being treated with L-Dopa and the Dopa decarboxylase inhibitor, α-methyldopahydrazine (MK-485), were analyzed. New constituents (compared with normal subjects not receiving the

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[8] Sharpless, N. S., and D. S. McCann. "DOPA and 3-O-Methyldopa in Cerebrospinal Fluid of Parkinsonism Patients During Treatment with Oral L-DOPA." *Clin. Chim. Acta 31*, 155 (1971).

[9] Cotzias, G. C., P. S. Papavasiliou, and R. Gellene. "Modifications of Parkinsonism -- Chronic Treatment with L-DOPA." *N. Engl. J. Med. 280*, 337 (1969).

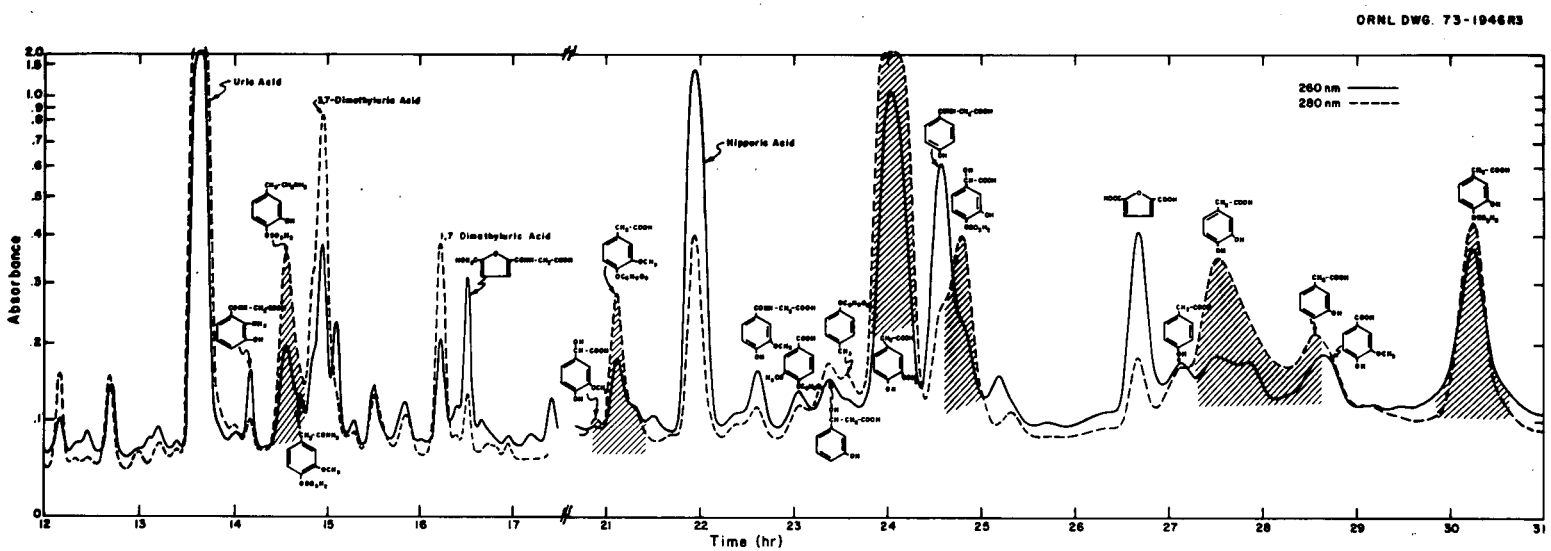

Fig. III.B.8. Elution Profile Observed on the UV Analyzer for a Patient with Parkinson's Disease Receiving 4 g of L-Dopa Every 24 hr. New and intensified peaks apparently due to the exogenous drug treatment are shaded.

Table III.B.5. Estimated Urinary Excretion of Metabolites by Parkinsonian Patients Treated with L-Dopa

Metabolite	Estimated Excretion (mg/24 hr)	
	Patient 1[a]	Patient 2[b]
Dopamine sulfate[c]	151	112
3-Methoxy-4-glucuronosidophenylacetic acid[c]	72	56
Vanillactic acid[d]	e	50
Homovanillic acid	1748	802
3,4-Dihydroxymandelic acid (sulfate conjugate)[c]	158	66
3,4-Dihydroxyphenylacetic acid	177	424
3,4-Dihydroxyphenylacetic acid (sulfate conjugate)[c]	147	e

[a]Subject ingesting 4 g/24 hr of L-Dopa.

[b]Subject ingesting 4.9 g/24 hr of L-Dopa.

[c]Conjugate quantified using molar absorptivity of the unconjugated compound.

[d]Quantified using the molar absorptivity of 4-hydroxyphenyllactic acid at 280 nm, 1226 liters $mole^{-1}$ cm^{-1}.

[e]Not detectable.

drug) and those normal constituents whose excretion was apparently increased by the combination drug treatment included dopamine sulfate, β-(3-methoxy-4-hydroxyphenyl)hydracrylic acid, homovanillic acid and its glucuronide, vanillactic acid, and the glycine conjugate of 3-methoxy-4-hydroxycinnamic acid. The excretion of the compound β-(3-hydroxyphenyl)hydracrylic acid, normally present in all urinary chromatograms, also appeared to be elevated; however, in the absence of a control urine without drugs, this could not be verified. Quantification data for a number of these compounds are shown in Table III.B.6.

Differences were noted among the three subjects on the combination drug treatment, and also between these subjects and those receiving only L-Dopa. An unknown chromatographic peak eluting at 34 hr on the uv chromatogram was significant and common to the three subjects receiving the combination drug therapy. It has not been observed in samples from normal subjects or from the two subjects receiving only L-Dopa. Identification of this compound and others may yield new information concerning the in vivo metabolism of L-Dopa in the presence of a decarboxylase inhibitor.

III.B.4.c. Aromatic Acids Excreted by Children with Neurological Disorders

Urine samples from a number of children with neurological seizures, infantile autism, extreme hyperactivity, and mental retardation have been analyzed on the UV Analyzer. There have been several similarities in elevated excretion of certain aromatic acids for a number of these children when compared with clinically normal children of approximately the same age. Various acid metabolites of phenylalanine and tyrosine were detected and quantitatively measured (Table III.B.7). Two of the uv chromatograms have a relatively strong chromatographic peak eluting at about 25.5 hr with maximum uv absorbance at 270 nm. This new compound has also been observed in chromatograms of patients afflicted with chronic leukemia, synovial sarcoma, embryonic neoplasia, malignant carcinoid, multiple myeloma, and Lesch-Nyhan syndrome. This peak has been identified as α-methoxy-α-(3-methoxy-4-hydroxyphenyl)acetic acid (trivially named α-methoxyhomovanillic acid), a homolog of VMA. Its pathologic significance or metabolic origin is unknown.

Table III.B.6. Estimated Rates of Excretion of Dopa Metabolites by Patients with Parkinson's Disease Receiving L-Dopa Plus α-Methyldopahydrazine[a]

Metabolite	Estimated Excretion Rate (mg/24 hr)		
	Patient 3[b]	Patient 4[b]	Patient 5[c]
Dopamine sulfate[d]	53	40	e
3-Methoxy-4-glucuronosidophenyl-acetic acid	f	f	f
Vanillactic acid[g]	213	38	42
Homovanillic acid	194	177	69
3,4-Dihydroxyphenylacetic acid	e	e	e
3,4-Dihydroxymandelic acid (sulfate conjugate)	e	13	e
3-Methoxy-4-hydroxycinnamic acid (glycine conjugate)[h]	4.5	e	8.3

[a]Each subject received 300 mg.

[b]Subject received L-Dopa at a dosage level of 2 g/24 hr.

[c]Subject received L-Dopa at a dosage level of 1.4 g/24 hr.

[d]Compound quantified using the molar absorptivity of the free amine.

[e]Not detectable by LC.

[f]Compound present but not quantified because of peak overlap.

[g]Quantified using the molar absorptivity for 4-hydroxyphenyllactic acid at 280 nm, 1226 liters $mole^{-1}$ cm^{-1}.

[h]Quantified using the molar absorptivity for 3-methoxy-4-hydroxycinnamic acid at 290 nm, 13,700 liters $mole^{-1}$ cm^{-1}.

Table III.B.7. Estimated Rates for Excretion of Some Acidic Metabolites by Three Children with Neurological Disorders and Three Normal Children

Compound	Estimated Excretion Rate (mg/24 hr)			
	Neurological Seizures, Age 9[a]	Infantile Autism, Severe, Age 8[b]	Infantile Autism, Mild, Age 8[c]	Normal Children, Ages 12, 9, and 8
β-(3-Hydroxyphenyl)hydracrylic acid[d]	42	26	35	12.5 (5.7)[e]
α-Methoxyhomovanillic acid[f]	70	42	[g]	[g]
4-Hydroxyphenyllactic acid	54	[d]	44	30.1 (14.4)[e]
4-Hydroxyhippuric acid[h]	38	14	12	7.6 (2.2)[e]
4-Hydroxyphenylacetic acid	31	40	9	10.0 (2.2)[e]

[a]Child on a low-purine diet which was also low in phenylalanine and tyrosine (1 to 2 g/24 hr). Drugs, 950 mg of allopurinol and 7 1/2 mg of phenobarbital.

[b]Drug, 5-hydroxytryptophan.

[c]Drug, allopurinol.

[d]Quantified using an estimated molar absorptivity of 1000 liters $mole^{-1}$ cm^{-1} at 270 nm.

[e]Standard deviation in parentheses.

[f]Quantified using an estimated molar absorptivity of 2700 liters $mole^{-1}$ cm^{-1} at 270 nm (VMA model).

[g]Not detectable.

[h]Quantified using molar absorptivity of 4-hydroxybenzoic acid at 260 nm, 11,000 liters $mole^{-1}$ cm^{-1}.

III.B.4.d. Metabolic Profiles for Children with Rare Diseases

Samples from children with relatively rare diseases were analyzed on the UV Analyzer with the cerate oxidimetric detector. The results from the study of the three diseases, eosinophilic granuloma, cystic fibrosis (and infectious hepatitis), and Henoch-Schönlein disease, are summarized in Table III.B.8.

III.B.4.e. Urinary Metabolites of 4-Hydroxyacetanilide

High-resolution liquid chromatographic studies on a number of different patients receiving the drug Tylenol (4-hydroxyacetanilide) have shown a marked similarity in the patterns of metabolites. Dual column monitors, uv photometers, and cerate oxidimetric detectors were used. A representative chromatogram illustrating the eight resulting metabolites of Tylenol is shown in Fig. III.B.9. The metabolites which have been identified include 3-methoxy-4-hydroxyacetanilide and its glucuronide and sulfate conjugates, and 4-hydroxyacetanilide and its glucuronide and sulfate conjugates. Two additional metabolites have also been associated with this medication (labeled U-A and U-B in Fig. III.B.9); however, they have not yet been identified.

Apparently the major metabolite of Tylenol therapy is the sulfate of 4-hydroxyacetanilide followed by its glucuronide. It is apparent that large amounts of conjugated 3-methoxy-4-hydroxyacetanilide are produced with the relative order of conjugation being sulfate > glucuronide > free compound. Burtis et al.[10] suggested that this metabolite was not normally observed after phenacetin ingestion; however, our data indicate that it is a normal metabolite of 4-hydroxyacetanilide.

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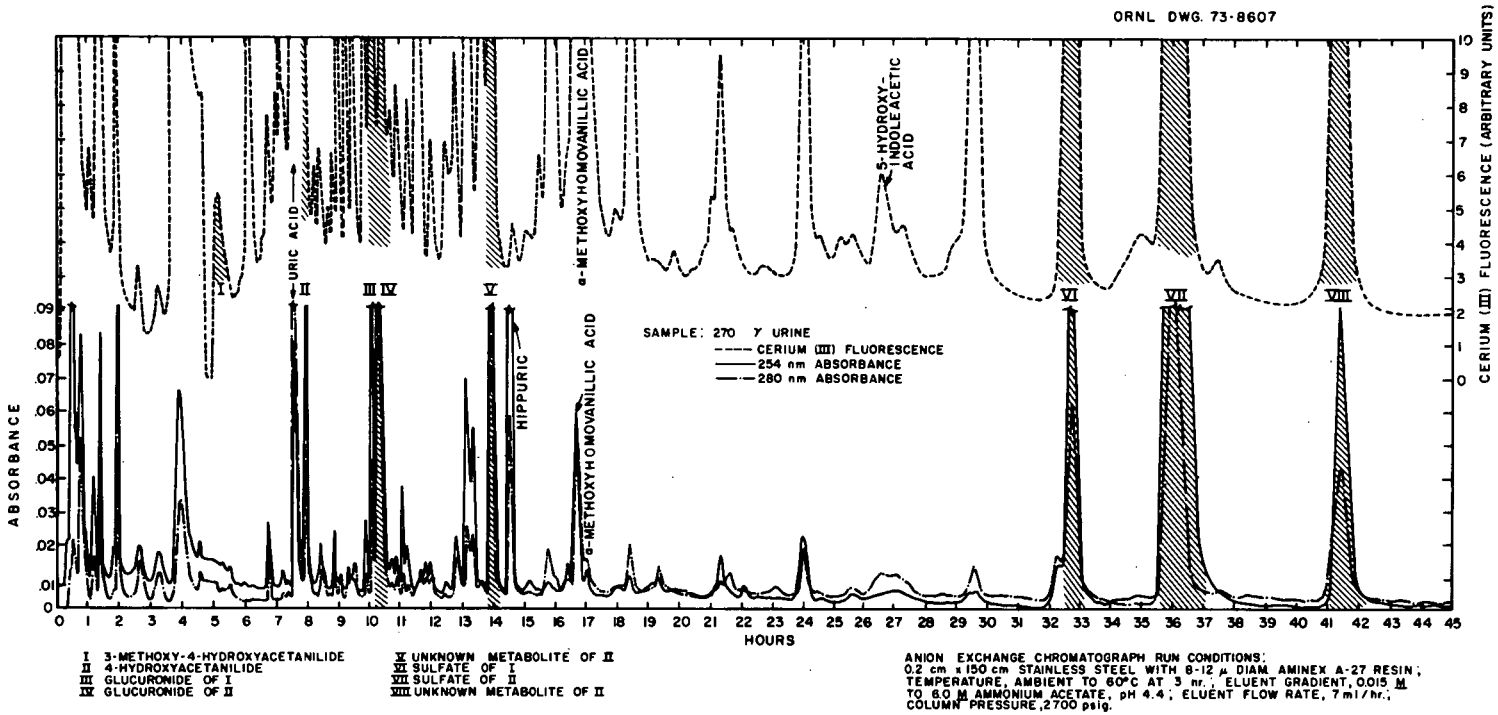
[10] Burtis, C. A., W. C. Butts, and W. T. Rainey, Jr. "Separation of the Metabolites of Phenacetin in Urine by High-Resolution Anion Exchange Chromatography." *Am. J. Clin. Pathol.* *53*, 769 (1970).

Table III.B.8. Compounds[a] Elevated in Patients with Rare Pathologic Conditions

Eosinophilic Granuloma	Cystic Fibrosis with Infectious Hepatitis	Henoch-Schönlein Disease
4-Glucuronosidotoluene[b]	4-Hydroxyphenylacetic acid	4-Hydroxyphenylpyruvic acid
Homovanillic acid[b]	β-(3-Methoxy-4-hydroxyphenyl)-hydracrylic acid	4-Hydroxyphenyllactic acid
VMA or α-ketoglutaric acid	4-Hydroxymandelic acid	Tyrosine
4-Hydroxyphenylacetic acid[b]	4-Glucuronosidotoluene	4-Cresol glucuronide
5-Hydroxyindoleacetic acid	4-Hydroxybenzoic acid, glycine conjugate	4-Hydroxymandelic acid
α-Methoxyhomovanillic acid[b]	4-Hydroxyhippuric acid, glycine conjugate	2 Substituted cinnamic acids

[a]Compounds separated and quantitatively determined by high-resolution liquid chromatography.

[b]Decreased amounts excreted after treatment.



CHILD WITH EOSINOPHILIC GRANULOMA, BEFORE TREATMENT, RECEIVING ANALGESIC MEDICATION

Fig. III.B.9. Urinary Chromatogram on High-Resolution Liquid Chromatographic Analyzer Showing Tylenol Metabolites.

III.C. PUBLICATIONS

Author(s)	Title	Publication
Burtis, C. A., W. F. Johnson, J. C. Mailen, J. B. Overton, T. O. Tiffany, and M. B. Watsky	"Development of an Analytical System Based Around a Miniature Fast Analyzer"	*Clin. Chem. 19*, 895 (1973)
Burtis, C. A., et al.	*Gravity Zero (GØ) Analytical Clinical Laboratory System Annual Progress Report for the Period Aug. 1 to Oct. 31, 1972*	ORNL-TM-4027
Burtis, C. A.	*Gravity Zero (GØ) Analytical Clinical Laboratory System Annual Progress Report for the Period. Apr. 15, 1972, to May 1, 1973*	ORNL-TM-4225
Chilcote, D. D., and C. D. Scott	"Mathematical Analysis of Normalization Techniques Used in Chromatography"	*Anal. Chem. 45*, 721 (1973)
Chilcote, D. D., and J. E. Mrochek	"Rapid Chromatographic Analysis of Nucleosides and N-Bases in Physiologic Fluids"	*Anal. Letters 6*, 531 (1973)
Chilcote, D. D.	"Computer Technique for Identification of Chromatographic Peaks"	*Clin. Chem. 19*, 826 (1973)
Katz, S., W. W. Pitt, Jr., and G. Jones, Jr.	"Sensitive Fluorescence Monitoring of Aromatic Acids After Anion-Exchange Chromatography of Body Fluids"	*Clin. Chem. 8*, 817-20 (1973)

(Publications - continued)

Author(s)	Title	Publication
Mailen, J. C., J. B. Overton, C. A. Burtis, and C. D. Scott	"Techniques for Fabrication and Assembly of Rotors for Use in a Miniature Fast Analyzer"	*Anal. Letters 6*, 245 (1973)
Mrochek, J. E., S. R. Dinsmore, and D. W. Ohrt	"Monitoring Phenylalanine-Tyrosine Metabolism by High-Resolution Liquid Chromatography"	*Clin. Chem. 19*, 927 (1973)
Neel, J. V., T. O. Tiffany, and N. G. Anderson	*Approaches to Monitoring Human Populations for Mutation Rates and Genetic Disease in Chemical Mutagens*	Chap. 28 in *Environmental Chemical Mutagens*, Vol. 3, ed. by A. Hollaender, pp. 105-50. New York: Plenum, 1973
Scott, C. D., and C. A. Burtis	"A Miniature Fast Analyzer System"	*Anal. Chem. 45*, 327-A (1973)
Scott, C. D.	"Health Care Delivery and Advanced Technology"	*Science 180*, 1339 (1973)
Tiffany, T. O., C. A. Burtis, J. C. Mailen, and L. H. Thacker	"A Dynamic Multicuvette Fluorometer-Spectrophotometer Based on the GeMSAEC Fast Analyzer Principle"	*Anal. Chem. 45*, 1716-23 (1973)
Tiffany, T. O., M. B. Watsky, C. A. Burtis, and L. H. Thacker	"Fluorometric Fast Analyzer: Some Applications to Fluorescence Measurements in Clinical Chemistry"	*Clin. Chem. 19*, 871-82 (1973)
Tiffany, T. O., D. D. Chilcote, and C. A. Burtis	"The Determination of Kinetic Parameters by Use of a Small-Computer Interfaced Fast Analyzer -- An Addition to Automated Clinical Enzymology"	*Clin. Chem. 19*, 909-18 (1973)

(Publications - continued)

Author(s)	Title	Publication
Waalkes, T. P., S. R. Dinsmore, and J. E. Mrochek	"Urinary Excretion by Cancer Patients of the Nucleosides, N^2,N^2-Dimethylguanosine, 1-Methylinosine, and Pseudouridine"	*J. Natl. Cancer Inst.* 51, 271 (1973)

III.D. ORAL PRESENTATIONS

Speaker and Coauthor(s)	Title	Place Presented
Anderson, N. G.	Position Paper: Multicomponent Analysis	Conf. on the Analyses of Multi-Component Mixtures and Their Application to Health Related Problems, Washington, D. C., June 11-12, 1973
Burtis, C. A., W. F. Johnson, J. C. Mailen, J. B. Overton, T. O. Tiffany, and M. B. Watsky	Development of an Analytical System Based Around a Miniature Fast Analyzer	Fifth Annual Symposium on Advanced Analytical Concepts for the Clinical Laboratory, Oak Ridge, Tenn., Mar. 15-16, 1973
Burtis, C. A.	The Centrifugal Fast Analyzer	Annual Convention of the Arkansas Society for Medical Technology and American Medical Technologists, Little Rock, Ark., Apr. 12, 1973
Burtis, C. A.	Miniature Laboratory System for Space Flights and Health Care Delivery	Eighth Annual Meeting of the Association for the Advancement of Medical Instrumentation, Washington, D. C., Mar. 23, 1973
Burtis, C. A.	Automated Clinical and Serological Analysis for Large-Scale Processing	Annual Meeting of the Tennessee Public Health Association and Conference of Tennessee Public Health Workers, Gatlinburg, Tenn., Mar. 28, 1973

(Oral Presentations - continued)

Speaker and Coauthor(s)	Title	Place Presented
Burtis, C. A.	Applications of a GeMSAEC Fast Analyzer	Department of Pharmacology, University of California at San Francisco, San Francisco, Calif., July 5, 1973
Chilcote, D. D.	A Mathematical Analysis of Normalization Techniques Used in Chromatography	Eighth International Symposium on Advances in Chromatography, Toronto, Canada, Apr. 16-19, 1973
Chilcote, D. D.	An Identification Scheme for Chromatographically Separated Constituents That Can Be Used with a Small Computer	Fifth Annual Symposium on Advanced Analytical Concepts for the Clinical Laboratory, Oak Ridge, Tenn., Mar. 15-16, 1973
Chilcote, D. D.	The Indole Analyzer: A Simple Sensitive Liquid Chromatographic Analyzer for Naturally Occurring Indoles in Body Fluids	Eighth Annual Meeting, Association for the Advancement of Medical Instrumentation, Washington, D. C., Mar. 21-24, 1973
Chilcote, D. D.	The Indole Analyzer: A Simple, Sensitive Liquid Chromatographic Analyzer for Naturally Occurring Indoles in Body Fluids	165th ACS National Meeting, Dallas, Tex., Apr. 8-13, 1973
Chilcote, D. D.	Computer Assisted Chromatographic Analysis of Body Fluid Samples	75th National Meeting of AIChE: Symposium of Engineering Methods in Medical Diagnostic Techniques, Detroit, Mich., June 3-6, 1973

(Oral Presentations - continued)

Speaker and Coauthor(s)	Title	Place Presented
Katz, S., W. W. Pitt, Jr., and G. Jones, Jr.	Sensitive Fluorescence Monitoring of Aromatic Acids After Anion-Exchange Chromatography of Body Fluids	Fifth Annual Symposium on Advanced Analytical Concepts for the Clinical Laboratory, Oak Ridge, Tenn., Mar. 15-16, 1973
Mailen, J. C.	The Development of a Miniature Fast Analyzer	75th National Meeting of AIChE: Symposium of Engineering Methods in Medical Diagnostic Techniques, Detroit, Mich., June 3-6, 1973
Mrochek, J. E.	Monitoring Phenylalanine-Tyrosine Metabolism by High-Resolution Liquid Chromatography	Fifth Annual Symposium on Advanced Analytical Concepts for the Clinical Laboratory, Oak Ridge, Tenn., Mar. 15-16 1973
Mrochek, J. E.	High-Resolution Liquid Chromatography - A New Analytical Tool for the Clinical Chemist	ACS Symposium, "Newer Analytical Techniques in Clinical Chemistry," at Regional Meeting of ACS, Cleveland, Ohio, May 14, 1973
Mrochek, J. E.	Liquid Chromatographic Analysis of Urinary Nucleosides in Normal and Malignant States	ACS Symposium on Analytical Advances in Clinical and Medicinal Chemistry at National ACS Meeting, Chicago, Ill., Aug. 30, 1973

(Oral Presentations - continued)

Speaker and Coauthor(s)	Title	Place Presented
Scott, C. D.	Introduction to the Fifth Annual Symposium on Advanced Analytical Concepts for the Clinical Laboratory	Oak Ridge National Laboratory, Oak Ridge, Tenn., Mar. 15, 1973
Scott, C. D.	High Resolution Liquid Chromatography as Applied to the Separation of Complex Biochemical Mixtures	Iowa State University, Ames, Iowa, Apr. 9, 1973
Scott, C. D.	High Resolution Liquid Chromatography as Applied to the Separation of Complex Biochemical Mixtures	3-M Company, St. Paul, Minn., Apr. 11, 1973
Scott, C. D., and N. E. Lee	Use of Sequential Columns of Microreticular and Pellicular Ion-Exchange Resins in the High-Resolution Separation of Complex Biochemical Mixtures	International Symposium on Column Liquid Chromatography, Interlaken, Switzerland, May 4, 1973
Scott, C. D.	Recent Developments in Fast Analyzer Systems	University of Birmingham, Birmingham, England, May 7, 1973
Scott, C. D.	High Resolution Liquid Chromatography	University of Salford, Salford, England, May 8, 1973
Scott, C. D., and W. W. Pitt, Jr.	Advances in the Development of High Resolution Liquid Chromatography as Applied to Analyses of Physiologic Fluids	75th National Meeting of AIChE: Symposium of Engineering Methods in Medical Diagnostic Techniques, Detroit, Mich., June 3-6, 1973

(Oral Presentations - continued)

Speaker and Coauthor(s)	Title	Place Presented
Scott, C. D.	Fast Analyzer Systems	American Society for Pharmacology and Experimental Therapeutics, East Lansing, Mich., Aug. 23, 1973
Tiffany, T. O., M. B. Watsky, and C. A. Burtis	Potential Use of the Fluorometric Fast Analyzer for Enzymatic and Immunological Assays in the Clinical Laboratory	Fifth Annual Symposium on Advanced Analytical Concepts for the Clinical Laboratory, Oak Ridge, Tenn., Mar. 15-16, 1973
Tiffany, T. O., D. D. Chilcote, and C. A. Burtis	Determination of Kinetic Enzyme Parameters for General Clinical Use, Genetic Screening, and Biomedical Research Through the Use of a Small Computer-Interfaced Fast Analyzer	Fifth Annual Symposium on Advanced Analytical Concepts for the Clinical Laboratory, Oak Ridge, Tenn., Mar. 15-16, 1973
Tiffany, T. O.	The Fluorescent Fast Analyzer	Spring Meeting, Southeast Section of the American Association of Clinical Chemists, Gatlinburg, Tenn., Apr. 5-6, 1973
Tiffany, T. O.	Recent Developments in Fast Analyzers	Joint Spring Meeting CSCC and AACC (Upstate N. Y. Section), Hamilton, Ontario, Canada, May 3-4, 1973

(Oral Presentations - continued)

Speaker and Coauthor(s)	Title	Place Presented
Tiffany, T. O., C. A. Burtis, and M. B. Watsky	The Use of the Fluorometric Fast Analyzer for Measuring Enzyme Activity (A Discussion of Direct Referencing of Fluorescence Assays)	25th National Meeting of the American Association of Clinical Chemists, New York City, July 15-20, 1973

IV. MEDICAL INSTRUMENTATION

G. D. James,[1] W. J. McClain, D. A. Ross, M.D., and P. R. Bell

We have produced the first "Family Image" using information accumulated in the course of the ORAU-sponsored gallium group studies. This first synthetic patient image confirms our predictions that such images would provide an objective, quantitative test of the truthfulness of image processing methods (see ORNL-4867). This family image, resembling a patient with Hodgkin's disease, has been used to test the validity of the antiscatter and antipenetration processing. The tests show that the processing produces a very great reduction in the difference between an ideal, noise-free image and the image corresponding to a clinical scan. A visual inspection of the unobtainable ideal image and the processed image shows that both the scatter and the statistical fluctuations have been reduced in effect.

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[1]Exchange Staff Member from United Kingdom Atomic Energy Authority, Harwell.

IV.A. INTRODUCTION

A considerable amount of effort has been and continues to be expended in efforts to improve the quality and readability of gamma-ray images. All gamma-ray patient images suffer from inadequate resolution and poor counting statistics. These defects are interrelated by difficult compromises in instrument design, image acquisition time, and tracer dose, so that great improvement is not expected. The images also contain a certain amount of false information derived from gamma-ray scattering in the patient and in the detecting instrument and from other effects. The obviously poor quality of the images and the relative intractability of the problem have created the widespread effort at data enhancement as a partial solution. Many image enhancement methods have been proposed and tried, but it is often difficult to decide upon their merits with actual patient images since the reality of evoked detail is difficult to establish.

IV.B. PHANTOMS

A "phantom" is often used to test imaging methods. Such phantoms often consist of simple geometric shapes filled with radioactive solution to represent hotter and colder regions and to provide edges, holes, etc., for testing the imaging process on a scan image of the phantom. These phantoms are generally rather simple and sometimes lead to results that are found to be unsatisfactory in use with patient images; for example, a smoothing function that best detects a medium-sized void in a uniform field of activity produces an image so smeared as to be unsatisfactory for general imaging. Phantoms, as generally prepared, are also too small and contain too little widespread activity to exhibit the full extent of scattering and septum penetration defects encountered in real patient images. A phantom large enough and with as much detail as an actual patient would be realistic enough but very difficult to prepare and use.

IV.C. A SYNTHETIC IMAGE PHANTOM

The idea of a wholly synthetic image as a comprehensive phantom is very attractive, since a computer is generally used in image processing.[1] A synthetic image can be readily adjusted to match the correct degree of resolution, gamma-ray scattering, collimator penetration, and counting statistics that correspond to the detector system, radionuclide, dose, and imagimg time of any particular scan. If done carefully, the resulting synthetic image will be indistinguishable from an actual patient image but will be more useful - although obscured by scattering, smearing, and statistical variation - are known. Of course, it is easy to produce any number of copies of this image differing only in the variation in the values in each picture element as though a new scan had been made of the same subject. Such a synthetic image would be of maximum value if it were made to contain all of the details frequently seen in a scan or camera image made with a specific radionuclide with a specific protocol for dose and imaging, since it could then serve the following additional conditions:

1. It could serve as a teaching aid for indoctrination of those not familiar with the nuclide in question.

2. It could demonstrate, by change of the counting statistics, the effects to be expected from a change of dose or counting times without actual trials.

3. It could demonstrate in an unambiguous way the changes to be expected by a change of system resolution (change of collimator) or changes in display format.

A synthetic image that thus represents a specific radionuclide we have named a "family image." Clearly, such an image also must represent a specific patient condition, but it represents all variations of resolution of

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[1]Synthetic organ images of this type have been described by A. G. Schultz et al. "Quantitative Assessment of Scanning-System Parameters." *J. Nucl. Med.* *11*(2), 61 (1970).

the camera or scanner and all variations in dose, imaging times, and detector sensitivities. Formation of a family image involves a number of steps:

1. Establishment of a group of separate basic data images of different organs at various depth zones. These basic images are added together in various proportions depending upon the degree of localization of the radionuclide, the body absorption of the radiations emitted, and the view being constructed. The image produced is the actual family image, although it does not resemble a patient scan until processed further.

2. The basic image must then be smeared by a convolution with a scattering function. The scattering function is obtained by comparing the measured point-source response of the imaging system to a source in air and in a water-scattering medium. The difference between these two responses, normalized to the same peak counting rate, is the scattering function. The convolution of the basic image with this function is added to the basic data, and this image is truncated at the body outline to form the second step image.

3. The second step image is then convolved with the response of the system to the point source in air to provide both the unsharpness due to the system resolution and the broader effect of septum penetration represented by the skirt of the response. This third step image represents a scan image without statistical variation.

4. The third step image is scaled by a common factor so that the individual picture elements contain the appropriate count values, and then each value is made to resemble a statistical count by a random number

process. The procedure used is the standard root-log-cosine method.[2] The statistical value N_1 corresponding to an element value N is given by

$$N_1 = N + (\cos 2\pi R_1)\ (-2N \ln R_2)^{\frac{1}{2}},$$

where R_1 and R_2 are random numbers in the range 0 to 1.

The result of step 4 is a final image that resembles an actual scan. It is only necessary to repeat step 4 to obtain an independent image similar in every detail except that each count is different as though a new scan had been made. The scaling factor may be changed to obtain an image representing a different imaging time, dose, or detector sensitivity.

The basic data images mentioned in step 1 are obtained by directly encoding, from anatomical charts, the outlines of the body and various organs. These lists are then converted into images with geometrically defined count levels by a program written in FOCAL.[3] The program also adds each basic image segment with appropriate multiplying factor to the image being formed. The total image occupies 16K picture elements (computer words) and is formed in 4 sections of 4K words each.

The convolutions required in steps 2 and 3 are performed by a slight modification of the ORSS-72 image processing program.[4,5] The randomization action of step 4 is done with a FOCAL program. All image manipulations are performed on a DEC PDP-8/I computer with 8K of core.

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[2] Box, G. E. P., and M. E. Muller. "A Note on the Generation of Normal Deviates." *Ann. Math. Stat. 28*, 610 (1958).

[3] FOCAL is the interpretive computer language of the Digital Equipment Corp.

[4] Bell, P. R., W. J. McClain, D. A. Ross, R. E. McKeighen, J. K. East, E. C. Moore, C. Lowell Edwards, and F. A. Goswitz. "A Computer-Based Scanning System." ORNL-TM-4038 (January 1973).

[5] Bell, P. R., et al. "ORSS-72: A Computer-Based, Dual-Data Rectilinear Scanning System." ORNL-TM-4045 (in press).

IV.D. PRELIMINARY FAMILY IMAGE

A first attempt has been made to produce a family image for ^{67}Ga. We hold no brief for the accuracy of this image, and it does not represent a normal patient; it rather resembles patients with some degree of Hodgkin's disease. Figure IV.D.1(A) shows the raw family image, while (C) shows a final image; (B) and (D) are the multicycle contrast enhancement images of (A) and (C) respectively. The point-source response used was that of an Ohio Nuclear scanner with 8-in. detectors and a medium-energy collimator.[6] The maximum count for the 1/4-in.-square picture elements was 200 at the highest point in the liver. It resembles rather well many scans we have seen on this instrument except that the structural details of the arms have not been put in below the axilla.

This family image was also formed, as in Fig. IV.D.1(C) with a maximum count per element of 100, and the resultant image was then processed by the ORSS-72 antiscattering and antipenetration routine to test its ability to recover the detail obscured by the statistical noise and the scatter and penetration defects. The product of the processing was then smoothed slightly by a Gaussian-shaped matrix averager. To secure a quantitative comparison, the original family image was convolved with the optical resolution function of the Ohio Nuclear collimator without the addition of any statistical noise. This comparison image after the same matrix smoothing, displayed in Fig. IV.D.2(A), is the ideal unreachable result toward which the processing is directed. The noisy scattered image is shown in (B), while the product of the processing is shown in (C). A chi-square test was made between the comparison image and the noisy scattered image and between the comparison and the processed images with results shown in Table IV.D.1. A comparison of the images shows the extent to which the evoked detail is trustworthy and allows a quantitative estimate of the amount of purely random and unreal detail evoked.

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[6] Collimator 85035-M with an optical cutoff resolution radius of 14 mm.

PHOTO 1190-73

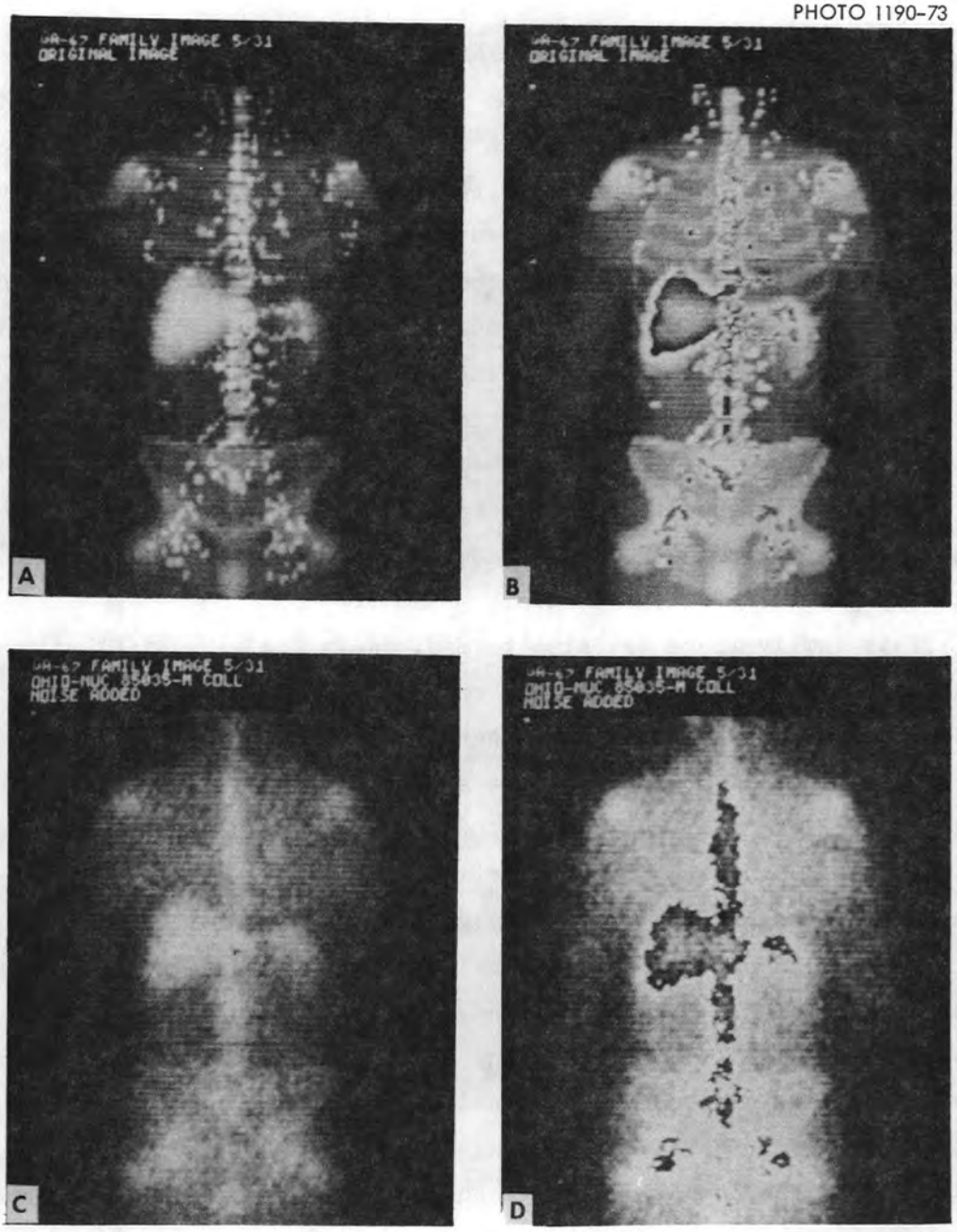

Fig. IV.D.1. Development of a Family Image. A "raw" family image is shown in (A). This image contains 12,000 picture elements. The arms below the axilla are incomplete with no bones. The multicycle contrast enhancement display of this image is shown in (B). The image has not been spread by any resolution function and contains no noise. The image in (C) has been convolved with the complete point source response of an Ohio Nuclear 8-in. detector with an 85035-M collimator. The source was ^{67}Ga placed at 7.5 cm depth in a 15-cm-deep water tank. Each picture element has been corrected to have the statistical noise corresponding to a count rate of 200 counts/picture element at the maximum of the liver. (D) is the corresponding MCCE display.

PHOTO 2171-73

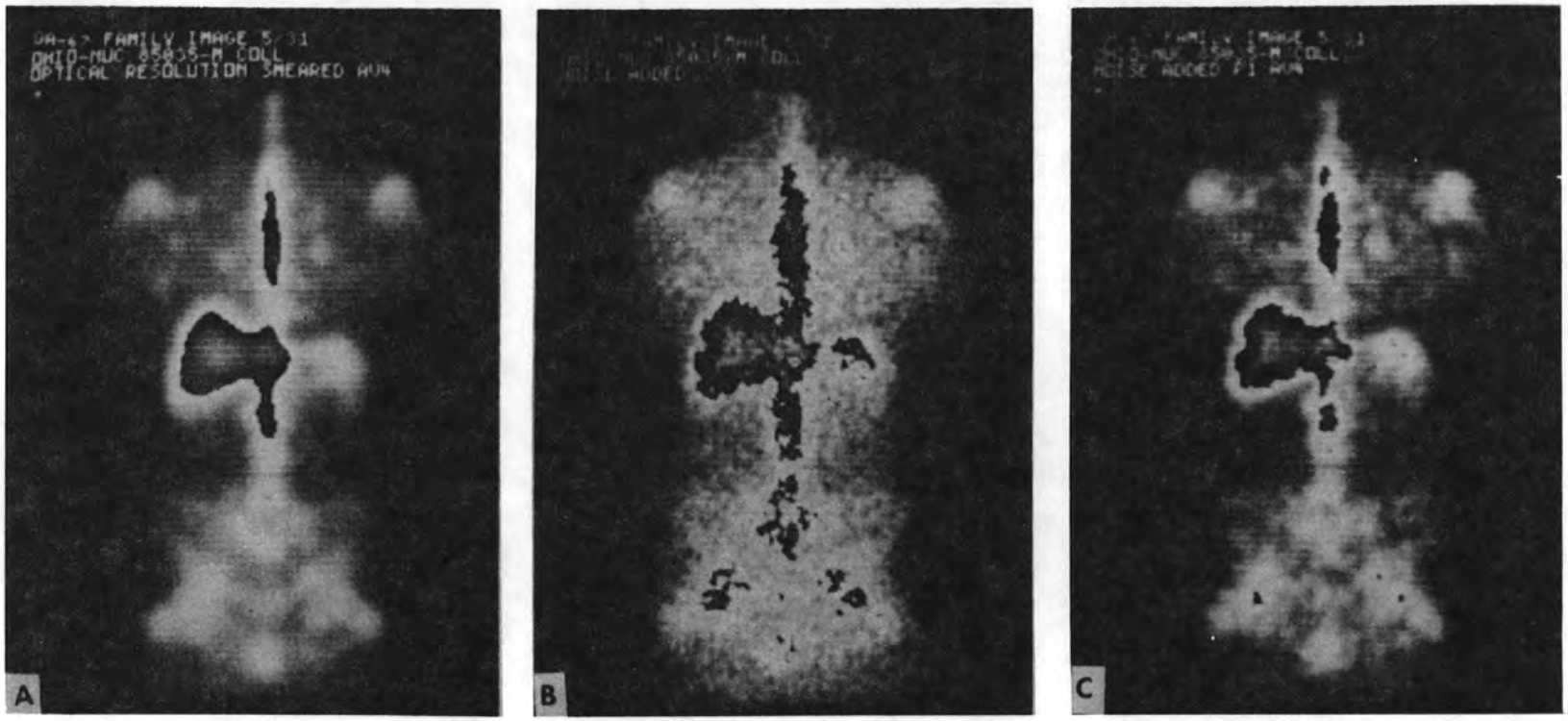

Fig. IV.D.2. Use of a Family Image to Test the Effectiveness of a Process. Image (A) is that of the noise-free family image of Fig. IV.D.1(A) convolved with the optical response function of the same collimator. The image has also been matrix averaged with a sharp averager function. Image (B) is like that of Fig. IV.D.1(D), except that the counting statistics correspond to a peak count of 100 counts/picture element at the maximum of the liver. Image (C) is that produced by processing the data of the center image by the anti-scatter and antipenetration process followed by the same matrix averager used for the image (A). Note that much of the detail visible in image (A) has been recovered.

Table IV.D.1.

Images Compared	χ^2 per Element
Noisy image with scatter and penetration defects vs optical resolution spread family image	3.97
Processed noisy and scattered image followed by averaging vs optical resolution spread image followed by averaging	0.60

IV.E. CONCLUSIONS

The family image has promise of providing a convenient mechanism for the testing of any image enhancement method that would be applied to a scan or camera image in digital form, or to any imaging process that can be simulated by a process performed on digital image data. It will also provide a vehicle for training technicians and for familiarizing others with specific radionuclide images. It can demonstrate the results to be expected for changes in instrument resolution, dose, imaging time (scan speed), and detector sensitivity. An extension of this method to other patient conditions and to other radionuclides seems worthwhile. This preliminary family image should be used to test the effectiveness of the many types of image processing methods now in use. Other family images should be prepared, and especially an attempt should be made to secure a "normal" patient image for ^{67}Ga.

IV.F. PUBLICATION

Author(s)	Title	Publication
Bell, P. R., W. J. McClain, D. A. Ross, R. E. McKeighen, J. K. East, and E. C. Moore	ORSS-72: A Computer-Based, Dual-Data Rectilinear Scanning System	ORNL-TM-4045 (August 1973)

IV.G. ORAL PRESENTATIONS

Speaker and Coauthor(s)	Title	Place Presented
Bell, P. R., W. J. McClain, D. A. Ross, R. E. McKeighen, J. K. East, and E. C. Moore	An Inexpensive Tape System for Remote Access of Dual-Scan Data	Southwestern Chap., Soc. Nucl. Med., New Orleans, La., Mar. 16-18, 1973
Bell, P. R., W. J. McClain, D. A. Ross, R. E. McKeighen, J. K. East, and E. C. Moore	The Reduction of Gamma-Ray Scatter and Septum Penetration in Scan Images	Southwestern Chap., Soc. Nucl. Med., New Orleans, La., Mar. 16-18, 1973
Bell, P. R., W. J. McClain, D. A. Ross, R. E. McKeighen, and J. K. East	ORSS Scanning System for Nuclear Medicine	1973 DECUS Spring Symp., Philadelphia, Pa., May 2-5, 1973
Bell, P. R., W. J. McClain, D. A. Ross, R. E. McKeighen, and J. K. East	Display Methods for Imaging Radioactivity Scans Using ORSS-72	1973 DECUS Spring Symp., Philadelphia, Pa., May 2-5, 1973
McClain, W. J.	Data Acquisition from a Rectilinear Scanner	A Workshop on the Fundamentals of Digital Data Processing in Clinical Nuclear Medicine, Miami Beach, Fla., Mar. 16, 1973

(Oral Presentations - continued)

Speaker and Coauthor(s)	Title	Place Presented
James, G. D., D. A. Ross, W. J. McClain, and P. R. Bell	A "Family Image" for Evaluation of Image Processing Methods	3rd Symp. on the Sharing of Computer Programs and Technol. in Nucl. Med., Miami, Fla., June 16-17, 1973

V. ASSOCIATED RESEARCH AND DEVELOPMENT

V.A. IMMUNOTECHNOLOGY

N. G. Anderson, Frances L. Ball, J. E. Caton, D. W. Holladay,
J. E. Attrill, Margaret Guess, and G. M. Henderson

Conventional methods for the purification of mixtures of proteins whose physicochemical differences are subtle generally result in incomplete and unsatisfactory separations of such mixtures. However, immunosubtractive chromatography utilizes the biological specificity of such macromolecules. This biological specificity involves localized regions in one macromolecule that is highly specialized in its recognition of some localized region of a second macromolecule. The interaction of this pair of macromolecules is very specific and there is little interference from other macromolecular species. Thus, immunosubtractive chromatography involves a stationary phase which consists of an inert support to which one of the interacting species has been bonded covalently. This covalently bonded macromolecule, or ligand, must be immobilized in such a way that its ability to recognize and interact with the other specific macromolecules is not impaired. Therefore, in successful immunosubtractive chromatography a sample mixture containing the macromolecule or macromolecules for which the column is specific is washed onto the column in the buffer used to equilibrate the column. The column adsorbs those macromolecules which the immobilized ligands on the column recognize. The remainder of the sample mixture is eluted from the column by the equilibrating buffer. The specifically adsorbed portion of the sample is then eluted from the column by changing conditions (pH, ionic strength) which break up the complex formed by the immobilized ligand and the macromolecules for which it is specific.

One of the most important features of immunosubtractive chromatography is that a small amount of a specific macromolecule can be utilized many times. Thus even if one has only a small amount of a specific macromolecular ligand, it can be utilized in an immunosubtractive chromatography procedure to prepare many times its equivalent of the macromolecules for which

it is specific. Now it is generally true that specific immunosorbents are available in very limited amounts; therefore, only small columns can be prepared. To prepare useful quantities of materials from such columns then requires many passes over such a column and much personal attention. To reduce the need for such attention an automated system was developed. A block diagram of the system is shown in Fig. V.A.1.

The operation of such a "Cyclum" can best be illustrated by following a sample through the system.

A set of three timers with accompanying microswitches activate the three solenoids which control the flow of buffer, sample, and eluting solution. At the beginning of a run, buffer solution is allowed to flow through the column until a straight base line is indicated on the recorder, indicating that the column is equilibrated. After a short period of time a timer activates a microswitch which closes solenoid 1 (buffer flow) and activates solenoid 2 (sample flow), allowing sample instead of buffer to be pumped to the column. An adjustable flow rate peristaltic pump maintains a constant flow rate set at 2 or 3 ml/min. Thus the period of time that solenoid 2 is open fixes the sample volume. After the desired period of sampling, the timer activates microswitches which close solenoid 2 and reopen solenoid 1. The sample solution is pushed over the column by buffer, and all proteins in the sample are passed through except the protein(s) which combines to the antibody or antigen that is covalently bound to the column. The unbound protein material is monitored as it passes through a photometer cell, and the peak is recorded on a strip chart recorder. From the photometer the column eluent is routed through a dialyzer system to a magnet solenoid-operated switch that directs it to the appropriate container.

After about 45 min, when the recorder base line shows the column to be free of all unbound protein, the timer again activates switches to change from buffer to an eluting solution which will break the antigen-antibody complex and allow the bound protein material to be eluted from the column.

This previously bound protein is passed through the dialysis system to remove the thiocyanate. From the dialyzer, it also is routed by the magnet solenoid switch to the proper collection container. After the second peak is collected, the clock timer again switches the eluting system

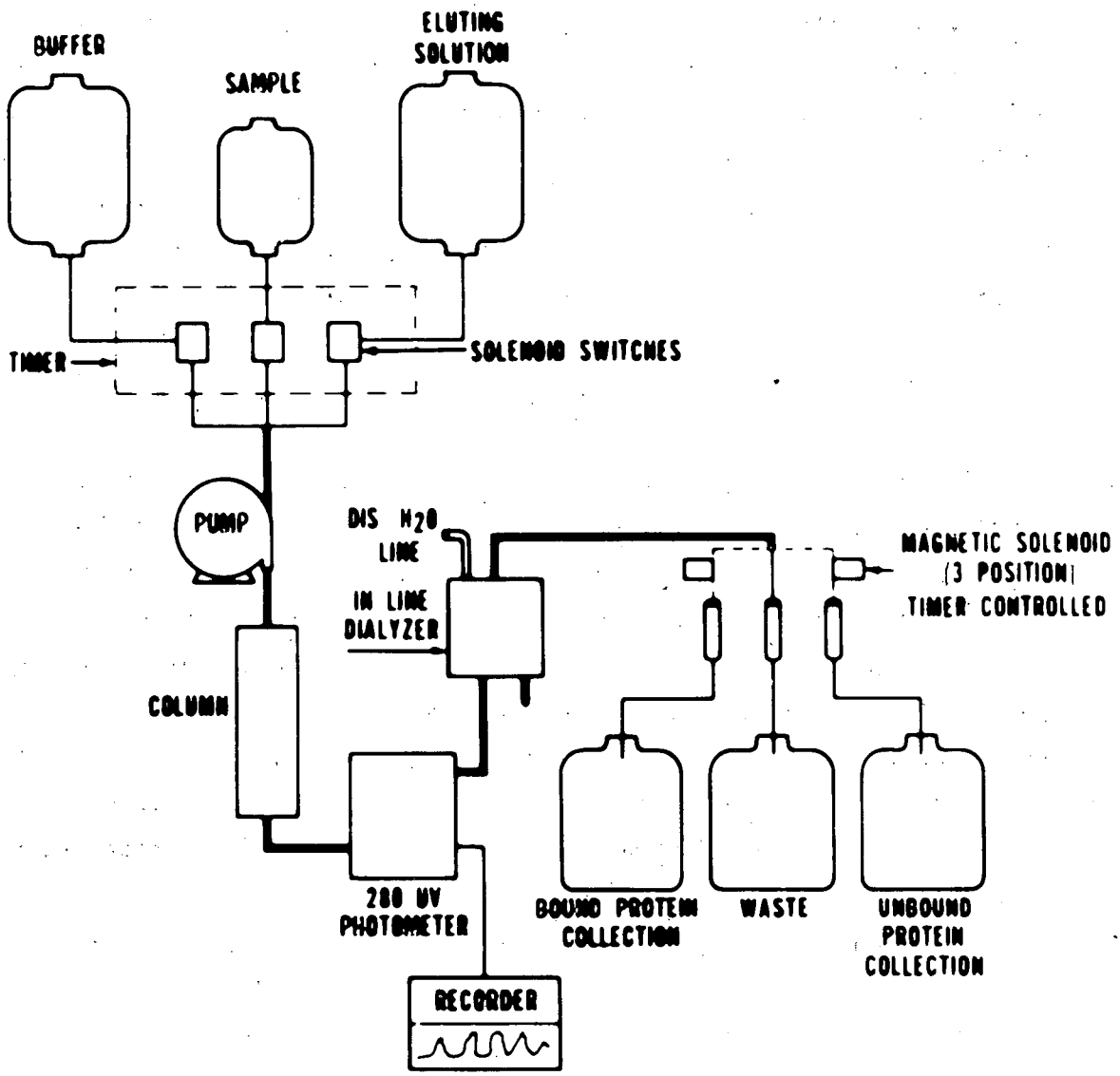

Fig. V.A.1. Schematic Diagram of a Cyclum.

back to buffer, and the column is cleaned and equilibrated for the next run. This cycle may be repeated as many times as necessary to separate and collect the required material from a given sample.

The total time required for a complete cycle is approximately 2 hr. The flasks of the bound and unbound protein are kept cold and removed as they become full. Concentration of the desired protein material to a usable volume may be accomplished by means of a pressure filtration through an Amicon PM 10 membrane filter.

The Cyclum has been employed in many different immunosubtractive chromatography systems. Some 30 rabbits and 8 goats are maintained to provide antibodies for immunosubtractive column preparation.

V.A.1. Preparation of Placental Antigens

An extensive project to prepare pure human placental antigens is being carried out using the Cyclums. Extracts are prepared from washed human placentas which are washed thoroughly, defatted, and cut into small (1 cm^2) pieces and frozen. Subsequently the frozen material is homogenized with 0.1 *M* phosphate buffer (pH = 7). The supernatant from this first homogenate is recovered, and a second homogenate with phosphate buffer is prepared. After recovery of the second supernatant, the remaining particulate material is homogenized with 3% aqueous NaCl and a third supernatant solution is recovered by centrifugation. The proteins of interest are recovered from each of the three supernatant solutions by a "salting-out" precipitation which makes each of the solutions 55% saturated with $(NH_4)_2SO_4$. The precipitates are recovered by dissolution in a minimum volume of 0.1 *M* phosphate buffer (pH = 7) and dialysis of the resulting solution against the same buffer. All three resulting protein solutions contain some antigens of interest; however, the concentration of these antigens decreases in the order of preparation.

Any remaining serum proteins can be removed from the placental extracts by passing them over an immunosubtractive column to which antibodies against human serum have been covalently bound.

We have used the unabsorbed fraction from this separation to immunize rabbits and a goat in order to obtain an immunochemical reagent for the non-serum proteins in human placental extract. This immunochemical reagent has been utilized for several different purposes: First, it has been used to prepare immunosubtraction columns for the direct subtraction of placental antigens from extracts. It has also proved to be a very useful antiserum for immunoelectrophoresis and double-diffusion experiments. It has shown reactivity with serum from cancer patients, with serum from pregnant women, and with protein concentrated from the urine of certain cancer patients. In addition, it has shown a marked absence of reactivity with the great majority of "normal" controls.

V.A.2. α-Fetoprotein

α-Fetoprotein has been isolated from human cord blood by a process not unlike the immunosubtractive chromatography scheme outlined above for placental antigens. As above, the normal serum proteins were subtracted from cord blood. Then the remaining cord blood constituents were used to immunize rabbits. The resulting antiserum proved to be a very good immunosubtractive reagent for column preparation, double-diffusion analytical work, and immunoelectrophoresis.

V.A.3. Cancer Antigens

Anti-normal human serum columns have also been used to subtract the "normal" human serum components from cancer serum. After exhaustive subtraction, several unusual proteins remain in the concentrate of the unsubtracted fraction. These proteins have been characterized by electrophoresis and have been used to prepare antisera in rabbits.

V.A.4. Pregnant Antigens

Serum drawn from women at term pregnancy has been treated in the same manner as cancer serum (described above).

V.A.5. Immunosubtraction with Antibodies from Pregnant Women

Immunosubtractive column packings were prepared using the immunoglobulin fraction of serum drawn from women at term pregnancy. This column appeared to have an affinity for some components of sera from cancer patients and for some components of placental extract, but it had little affinity for any of the components of normal human serum, of serum from women at term pregnancy, or of serum from cord blood. In addition, the column had little affinity for α-fetoprotein. Concentrates of the fractions for which this column had affinity are being studied by gel electrophoresis. However, at this time the study must be considered as being in its initial stages, and further analysis will be reserved for a later report.

V.A.6. "All But One" Serum Proteins[1]

The antibodies against a specific human serum protein were removed from a mixture of antibodies against all human serum proteins, with the resulting mixture of antibodies being an antiserum against "all but one" human serum proteins. The antibodies against the specific protein were removed by either of two methods: (1) by precipitating the specific antibodies from the mixture by simple addition of the specific protein; or (2) by covalently linking the specific serum protein to Sepharose 4B (trade name of Pharmacia Fine Chemicals, Inc.) and then employing the resulting Sepharose-protein complex in immunosubtractive chromatography to selectively remove the antibodies to that one protein from the antibody mixture. This mixture of antibodies against all human serum proteins except one is then used in immunosubtractive chromatography. With such a column packing only the "all but one" protein will be eluted by the equilibrating buffer when the column is charged with a sample of normal human serum. Such a scheme offers the possibility of the daily preparation of an antigen that is quite labile.

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[1] Attrill, J. E., J. E. Caton, D. W. Holladay, and N. G. Anderson. "Affinity Chromatography of Human Serum Using Antisera Against 'All But One' Human Serum Proteins." 165th ACS National Meeting, Dallas, Tex., Apr. 8-13, 1973. ANAL 32.

The "all but one" serum proteins can be recovered from the above schemes and used to immunize animals. Thus a source of "all but one" antiserum is available. The antigens for preparing an "all but one" antiserum have also been prepared by immunosubtractive chromatography using an antiserum against a specific human serum protein and subtracting that specific protein from a human serum sample. The preparation of human serum minus hemopexin is a good example of this procedure, and it also serves to demonstrate the utility of the Cyclum in making maximal use of a small quantity of antibody. In this case only enough antibody to react with the hemopexin in about 300 μl of human serum was available. However, a column prepared from this antibody was used to subtract the hemopexin from over 30 ml of human serum. The human serum was passed over the column five times, with each pass requiring about 30 cycles. The total time for the five passes was about 2 weeks. The actual operator time, however, was probably less than 2 hr. Similar serum-minus-one preparations were made for albumin, transferrin, and ceruloplasmin. Antisera to all these preparations have also been made. The antibodies from these antisera will be used in immunosubtractive chromatography to demonstrate both analytical and preparative schemes.

V.A.7. Immunosubtractive Electrophoresis

The specificity of antibodies has also been directly employed in analytical electrophoresis.[2] Here a band or bands may be subtracted with specific antibodies prior to electrophoresis. By subtracting or diminishing a band with a specific antibody, one has essentially identified that band in the resulting electrophoretic pattern when it is compared with a control. Thus such a technique retains the identification advantages of immunodiffusion or immunoelectrophoresis without sacrificing any of the much higher degree of resolution obtained by polyacrylamide gel electrophoresis.

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[2] Caton, J. E., D. W. Holladay, and N. G. Anderson. "Immunosubtraction Electrophoresis on Gradient Polyacrylamide Gels." 165th ACS National Meeting, Dallas, Tex., Apr. 8-13, 1973. ANAL 31.

V.A.8. Detection of Differences in Extracted Proteins of Soybean Varieties and Induced Mutants

A study to ascertain whether or not protein differences in extracts of different soybean flours could be detected by polyacrylamide gel electrophoresis was carried out by Kathy Lowry, ORAU Undergraduate Research Trainee from New College, Sarasota, Florida; her advisors were D. E. Foard (Biology Division) and J. E. Caton.

Six different flours were extracted into eight different solvents. Each extraction was carried out two ways, by sonication and by agitation. Thus, 96 extracts were prepared.

Of the eight solvents, a tris-glycinate buffer (pH 8.6) containing 2-mercaptoethanol extracted the most protein for electrophoretic analysis of the flours of soybean varieties Lee, Pickett, and Harosoy and three radiation-induced morphological mutants of Harosoy. Sonication extracted more protein than agitation. The only effect of type of solvent and of sonification vs agitation was in the amount of protein dissolved. Electrophoresis of seed proteins on gradient polyacrylamide slab gels detected previously unreported differences between Lee and Pickett varieties and revealed that two of the mutants have a pattern similar to each other, but different from the other mutant. The latter had a pattern similar to the parent Harosoy variety. Treatment of the extracts with sodium dodecylsulfate and 2-mercaptoethanol before electrophoresis on gels containing sodium dodecylsulfate (1) distinguished differences in major proteins or protein subunits of the two mutants that had similar patterns on gradient gels, and (2) revealed that the remaining mutant and the three varieties share the same proteins, differing only in the amounts of a given protein.

V.B. BLOOD CELL SEPARATORS FOR IN VIVO LEUKAPHERESIS

J. P. Breillatt, W. K. Sartory, C. J. Remenyik, C. D. West, D. D. Willis, Helen Vodopick, and J. N. Brantley

We have continued our theoretical studies to optimize the design of centrifugal blood cell separators. The final fabrication steps on the axial-flow separator have recommenced since the initiation of the NCI-AEC Interagency Agreement in September 1973. Design parameters for the radial-flow separator have reached the stage at which working drawings can be started. The conductivity/hematocrit detector system has been built and tested. This detector will be used to maintain the hematocrit in the packed red cell line at 0.70 through feedback control to the rotor drive motor. Thus the rotor velocity will become a function of the erythrocyte sedimentation rate in the donor blood -- a primary consideration in optimizing leukocyte recoveries from individual donors. Successful operation of a blood cell separator further requires that the red cell/plasma interface be located within a given region of the rotor cavity. The interface will be detected photometrically and its location controlled through feedback to the plasma return pump.

Maintenance of leukemia patients frequently requires transfusions of red cells and platelets. If leukocytes are not removed from these preparations, the patient may become immunologically sensitized to the leukocyte antigens present. It is not uncommon for leukemia patients to possess a broad spectrum of antileukocyte antibodies or leukoagglutinins. Individuals with high titers of these antibodies do not respond to granulocyte replacement therapy, and an antibiotic-resistant infection becomes life-threatening.

This situation might be partially prevented at the very onset of the disease by rigorously removing leukocytes and their antigens from all preparations for transfusion. Another approach is to select donors whose leukocyte antigens match those of the recipient, if they can be found.

We present a third approach, to remove from the prospective recipient's circulation those specific anti-leukocyte antibodies responsible for inactivating the granulocytes transfused from a given donor.

Granulocytes and monocytes are collected from human donors on columns of nylon wool (Leucopak, TM-Fenwal) inserted in an extracorporeal loop of their circulatory system. Studies elsewhere of the statistics of cell recovery and scanning electron micrographs of loaded and eluted fibers indicate that granulocytes and monocytes accumulate in layers of 3-4 deep on the nylon fibers, but that elution with citrated plasma and analogous solutions leaves the base layer of cells firmly attached to the fibers. This eluted Leucopak is generally discarded, representing a 20-40% loss of granulocytes. An eluted Leucopak also can be viewed as an affinity or immunoabsorption column specific for antibodies directed against the cells left on the fibers. After adequate washing, the Leucopak is included in an extracorporeal circuit of the infected patient possessing anti-leukocyte antibodies. The recipient's blood (or plasma phase) is passed through the Leucopak. When the level of circulating antibodies specific for the donor's leukocytes reaches an appropriate low titer, the previously eluted granulocytes can be transfused with enhanced probability of functional survival.

While we have emphasized the removal of circulating antibodies, it is possible that the sensitized lymphocytes of the recipient responsible for the cell-mediated response to the foreign leukocytes would also remain in the Leucopak with the immobilized donor cells.

V.C. HETEROGENEITY OF CARCINOEMBRYONIC ANTIGEN

J. W. Eveleigh

DEAE-cellulose chromatography of CEA prepared by conventional perchloric acid extraction and affinity chromatography has revealed the presence of several antigenically reactive components. At least eight distinct proteins have been isolated and preliminary characterization of these will be reported. It appears that the majority of the components have a similar molecular weight but differ in their overall ionic charge and amino acid composition. As commercial preparations of CEA reveal a similar heterogeneity, their significance in the current radioimmunological assay for serum CEA levels will be discussed.

Amino Acid Composition of CEA Components

Amino Acid	CEA Component					
	1_A	1_B	2	3	4_A	4_B
Lys	46.3	27.8	30.7	34.1	74.6	67.5
His	15.7	16.8	17.5	19.2	24.1	20.4
Arg	36.7	36.2	30.3	34.4	27.3	35.6
Asp	123.3	139.8	126.2	126.7	104.1	102.2
Thr	91.8	90.3	85.0	86.7	69.8	67.8
Ser	103.8	104.2	96.5	93.6	51.0	59.3
Glu	105.2	99.4	94.9	78.2	119.8	110.5
Pro	72.1	76.5	67.9	74.5	40.8	37.2
Gly	62.7	57.3	53.0	52.9	45.3	43.1
Ala	56.4	63.8	56.2	59.5	55.3	66.7
Cys/2	-	-	16.4	14.4	t	t
Val	71.4	77.8	66.7	64.5	52.5	52.9
Met	-	-	-	-	8.5	10.4
Iso	45.9	45.0	40.9	42.5	40.1	40.6
Leu	85.3	85.5	84.1	82.6	97.7	104.3
Tyr	-	-	34.4	32.6	22.6	22.7
Phe	24.4	24.6	23.6	28.5	53.1	50.0

[Abstract of a paper to be presented at the 3rd Conference on Embryonic and Fetal Antigens in Cancer, Nov. 4-7, 1973, at the Hyatt Regency Hotel, Knoxville, Tennessee.]

V.D. FUNCTIONAL ORGANELLES IN PROCARYOTES: POLYHEDRAL INCLUSIONS (CARBOXYSOMES) OF *THIOBACILLUS NEAPOLITANUS*

J. M. Shively, Clemson University; Frances L. Ball;
D. H. Brown, ORAU; and R. E. Saunders

The polyhedral inclusions of *Thiobacillus neapolitanus* have been isolated and found to contain ribulose-1,5-diphosphate carboxylase. It is proposed that these organelles, functioning in the fixation of carbon dioxide, be called carboxysomes.

[Abstract of a paper scheduled for publication in *Science*.]

V.E. ELECTRON MICROSCOPY OF THE CARBOXYSOMES (POLYHEDRAL BODIES) OF *THIOBACILLUS NEAPOLITANUS*

J. M. Shively, Clemson University; Frances L. Ball;
and Betty W. Kline

The carboxysomes of *Thiobacillus neapolitanus* are shown, by electron microscopy, to consist of a paracrystalline array of 10 nm particles surrounded by a "membrane." The particles have a center hole or depression and have been previously identified as ribulose-1,5-diphosphate carboxylase. The membrane is a monolayer approximately 3.5 nm thick.
[Abstract of a paper scheduled for publication in *Journal of Bacteriology*.]

V.F. PUBLICATIONS

Author(s)	Title	Publication
Anderson, N. G., D. W. Holladay, J. E. Caton, and L. B. Peterson	"Isolation of Tumor-Associated Macromolecules Using Immunosubtraction"	*Federation Proc. 32*, 961 (1973)
Anderson, N. G.	"Prospective Biology. Can the Course of Future Research and Development Be Foreseen with Sufficient Clarity To Allow Sensible Planning?"	*Biologie Prospective, II Colloque de Pont-a-Mousson*, 1972, L'Expansion Scientifique Francais, ed. pp. 555-560.
Attrill, J. E., J. E. Caton, D. W. Holladay, and N. G. Anderson	"Affinity Chromatography of Human Serum Using Antisera Against 'All But One' Human Serum Proteins"	*165th ACS Natl. Mtg., Dallas, Tex., Apr. 8-13, 1973*, ANAL 32
Brown, D. H., E. Carlton, B. Byrd, B. Harrell, and R. L. Hayes	"Rate-Zonal Centrifugation Procedure for Screening Particle Populations by Sequential Product Recovery Utilizing Edge-Unloading Zonal Rotors"	*Arch. Biochem. Biophys. 155*, 9-18 (1973)
Caton, J. E., D. W. Holladay, and N. G. Anderson	"Immunosubtractive Electrophoresis on Gradient Polyacrylamide Gels"	*165th ACS Natl. Mtg., Dallas, Tex., Apr. 8-13, 1973*, ANAL 31
Welton, T. A., F. L. Ball, and W. W. Harris	"Wiener Processing of Phase Contrast Electron Micrographs"	*Proc. 31st Ann. Mtg. Electron Microscopy Soc. Am., New Orleans, La., Aug. 14-17, 1973.* Baton Rouge, La.: Claitor's Publishing Division, p. 270

V.G. ORAL PRESENTATIONS

Speaker and Coauthor(s)	Title	Place Presented
Anderson, N. G.	Differentiation, Retrogenesis, and Cancer	VIIth Intern. Congr. of the Intern. Soc. of Developmental Biologists, Montreal, Canada, Aug. 19-24, 1973
Anderson, N. G.	Embryology, Retrogenesis, and Cancer	Karl August Forster Lecture, German Academy of Arts and Sciences, Mainz, May 4, 1973
Anderson, N. G., D. W. Holladay, J. E. Caton, and L. B. Peterson	Isolation of Tumor-Associated Macromolecules Using Immunosubtraction	57th Ann. Mtg., Fed. Am. Soc. Exptl. Biol., Apr. 15-20, 1973, Atlantic City, N. J.
Anderson, N. G.	Molecular Anatomy of Neoplasia	American College of Physicians Course in Medical Oncology and Chemotherapy, Univ. of Southern Calif. School of Medicine, Los Angeles, June 14-15, 1973
Anderson, N. G., and J. H. Coggin, Jr.	Molecular Mechanisms in Retrogenesis	Conf. on Paraneoplastic Syndromes, N. Y. Acad. Sci., New York City, Mar. 26-29, 1973
Attrill, J. E., J. E. Caton, D. W. Holladay, and N. G. Anderson	Affinity Chromatography of Human Serum Using Antisera Against "All But One" Serum Proteins	ACS 165th National Meeting, Dallas, Tex., Apr. 8-13, 1973

(Oral Presentations - continued)

Speaker and Coauthor(s)	Title	Place Presented
Breillatt, J. P.	Centrifugal Solutions to Biomedical Problems	Western Kentucky Section AIChE, Paducah, Ky., May 15, 1973
Caton, J. E., D. W. Holladay, and N. G. Anderson	Immunosubtraction Electrophoresis on Gradient Polyacrylamide Gels	ACS 165th National Meeting, Dallas, Tex., Apr. 8-13, 1973
Halsall, H. B.	The Zonal Centrifuge: Analytical Techniques and Pitfalls	Department of Biochemistry, Univ. of Tennessee, Knoxville, Mar. 28, 1973
Holleman, J. W.	Protein Purification by Immuno-adsorption	Engineering Foundation Conference, Henniker, N.H., Aug. 5-10, 1973
Moss, M. L., and W. W. Harris	Ultramicro Approach to Prenatal Diagnosis	ACS 165th National Meeting, Dallas, Tex., Apr. 8-13, 1973
Welton, T. A., F. L. Ball, and W. W. Harris	Wiener Processing of Phase Contrast Electron Micrographs	Electron Microscopy Society of America, 31st Ann. Mtg., New Orleans, La., Aug. 14-17, 1973

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