Table 2. Effect of added NaCl on the oxidation of ascorbate and coupled phosphorylation in turnip root mitochondria. Oxygen uptake and phosphate esterification were measured as described in the text. Each flask contained: tris ascorbate, 6 μmole; cytochrome *c*, 0.005 μmole; $MgSO_4$, 30 μmole; tris-ADP, 0.5 μmole; tris-PO_4, 4 μmole; sucrose, 600 μmole; tris-Cl (*p*H 7.0), 75 μmole; glucose, 50 μmole; NaCl as indicated; hexokinase, 0.2 units; and 0.5 ml of mitochondrial suspension. The total volume was 3.0 ml; the center well contained 0.2 ml of 5*N* KOH; the gas phase was air; and the temperature was 27°C. Values given are means of four determinations.

NaCl (mole/liter)	Oxygen uptake (μatom)	Phosphate esterified (μmole)	P/O
0	1.39	1.26	0.91
1×10^{-5}	1.43	1.26	.88
1×10^{-4}	1.55	1.24	.80
3×10^{-4}	1.59	1.10	.69
6×10^{-4}	1.65	1.02	.62
1×10^{-3}	1.70	0.97	.57
3×10^{-3}	1.67	.98	.58
6×10^{-3}	1.60	1.10	.69
1×10^{-2}	1.46	1.10	.75
1×10^{-1}	1.32	1.11	.83

$10^{-1}M$ NaCl, where the rates of both cytochrome *c* reduction and phosphorylation are diminished to about half of the control values.

The second site, separated from the other by the use of ascorbate as a reductant for cytochrome *c*, the oxidation of which is coupled to the phosphorylation, and molecular oxygen as a terminal acceptor for the electrons, is affected by NaCl in the manner detailed in Table 2. In this case, oxygen uptake is somewhat increased (about 20 percent) by $10^{-3}M$ NaCl, but phosphorylation is inhibited to a similar degree by the same concentration. The result is to reduce the P/O ratio from 0.91 to a value only slightly greater than ½ of the theoretical 1. Because the amount of mitochondria and the times of measurement differ for the experiments reported in Tables 1 and 2, it is not possible to calculate accurately the P/O ratios for the summation of the activity of both sites measured separately. But an estimation of these values, corrections being made for differing volumes, amounts of mitochondria, adenosine diphosphate and reaction time, gives results in general agreement with the data reported in Fig. 1, with the calculated P/O for the sum of both sites at $10^{-3}M$ NaCl being 80 percent higher than that for the preparations with no added NaCl as compared with a 50 percent increase in the P/O ratios actually observed in the experiments detailed in Fig. 1. It therefore seems reasonable to assume that the observed effect on phosphorylation when both sites are in operation is actually a summation of the effects of Na^+ observed on the two sites separately.

One might assume that the effect of Na^+ on phosphorylation may be related to the ($Na^+ + K^+$)-activated adenosine triphosphatases which have been reported from a variety of sources (*7*, *8*). A report of stimulation by K^+ of oxidative phosphorylation in brain mitochondria (*9*) has been explained on this basis. However, our evidence gives little support to such an assumption, particularly since the optimum concentration of Na^+ found here is one or two orders of magnitude lower than those reported for the adenosine triphosphatases. In addition, there appears to be no requirement for both cations. In experiments involving both Na^+ and K^+, the effect of the two ions in combination was simply additive.

Although it might be possible that Na^+ or other monovalent cations are required as an activator for the phosphorylation accompanying the reduction of cytochrome *c*, it seems improbable that the mechanism of this activation is through binding of the ion at a specific point, either the active site of the phosphorylating enzyme, or at an allosteric site. It is, of course, possible that the influence of the cations on the activity of both the phosphorylation sites studied is not exerted through an effect on the configuration of a single enzyme, but instead through influences on the architecture of the mitochondrion.

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References

1. E. Troug, K. C. Berger, O. J. Attoe, *Soil Sci.* **76**, 41 (1953).
2. P. F. Brownell and J. G. Wood, *Nature* **179**, 635 (1957).
3. A. Livine and Naomi Levin, *Plant Physiol.* **42**, 407 (1967).
4. R. T. Wedding and M. Kay Black, *ibid.* **37**, 364 (1962).
5. B. Borgström, H. C. Sudduth, A. L. Lehninger, *J. Biol. Chem.* **215**, 571 (1955).
6. G. F. Maley and H. A. Lardy, *ibid.* **210**, 903 (1954).
7. D. M. Blond and R. Whittam, *Biochem. Biophys. Res. Commun.* **17**, 120 (1964).
8. A. Pena-Diaz, C. Campillo-Serrano, M. Tuena, A. Gomez-Puyou, *Arch. Biochem. Biophys.* **106**, 461 (1964); F. J. Samaha and J. Gergely, *ibid.* **114**, 481 (1966).
9. A. R. Krall, Margaret C. Wagner, D. M. Gozansky, *Biochem. Biophys. Res. Commun.* **16**, 77 (1964).

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Cytotoxic Action of Stimulated Lymphocytes on Allogenic and Autologous Erythrocytes

Abstract. *Fowl erythrocytes are lysed when exposed to an excess of fowl blood lymphocytes in the presence of phytohemagglutinin. No significant cell damage is seen in the absence of phytohemagglutinin, or when the lymphocytes are replaced by malignant lymphoid cells, thymus cells, or nonlymphoid cells. The lymphocytes remain viable during the reaction. Differences in histocompatibility between lymphocytes and erythrocytes are not required. Autologous lymphocytes are cytotoxic to the same extent as allogenic lymphocytes over a wide range of experimental conditions.*

Lymphocytes are assumed to play an important role in tissue-damaging immune reactions, such as those occurring in delayed hypersensitivity, in various homograft and graft-versus-host interactions and in autoimmunity. Evidence for their possible direct participation in cell destruction comes from experiments in vitro in which antigen-carrying cells in tissue culture are damaged when exposed to lymphocytes from sensitized animals. The reactions are immunologically specific, do not require addition of complement, and are not correlated with titers of cytotoxic antibody in the blood of the lymphocyte donors (*1*). These reactions appear also when lymphocytes and target cells are of syngenic or even autologous origin (*2*). Furthermore, lymphocytes from normal donors may become cytotoxic for tissue culture cells, provided they have been stimulated by phytohemagglutinin (PHA) or antigen (*3–5*). Cytotoxicity requires living, immunologically competent lymphocytes whose energy-supplying metabolism is intact (*6*).

The use of nondividing target cells offers many advantages in studying the problem of the mechanism of lymphocyte cytotoxicity. We now report that fowl erythrocytes can easily be used for this purpose. In contrast to what has been found with tissue culture cells, lack of histocompatibility between lymphocytes from normal donors and fowl erythrocytes is not necessary for the PHA-induced cytotoxic reaction.

Twenty milliliters of blood from White Leghorn fowls (3 to 12 months old) were taken up in a syringe containing 100 I.U. of heparin in 3 ml of a 1:1 mixture of Hanks's balanced salt

solution (BSS) and 0.15*M* tris buffer, *p*H 7.4. The blood was incubated for 1 to 2 hours at 37°C in nonsiliconized centrifuge tubes. After centrifugation for 5 minutes at low speed, plasma and buffy coat were siphoned off with a pipette, care being taken not to remove polymorphs and monocytes attached to the walls of the tubes. The cells were washed twice in tris-buffered BSS by centrifugation at 1000 rev/min for 10 minutes, and were finally suspended in Parker's medium 199, containing penicillin and streptomycin (*7*, *8*), and 5 percent of either heat-inactivated fowl serum or fetal calf serum. The concentration of living lymphocytes, determined by supravital staining with 0.05 percent trypan blue, was kept at 20×10^6/ml until testing. Such samples were practically free from erythrocytes. They contained less than 2 percent polymorphs and monocytes. The cells were labeled with ^{51}Cr-chromate (Table 1).

For the cytotoxicity test, 0.5 ml of the lymphocyte suspension (25 to 100 $\times 10^5$ living cells) was mixed with 0.5 ml of erythrocytes (10^5 cells) in roller tubes. To this, 0.5 ml of the same medium, or medium supplemented with PHA, was immediately added (*7*, *8*). The tubes, equipped with loosely fitting screw caps, were incubated in duplicates in an atmosphere of 95 percent air and 5 percent CO_2. Cytotoxicity is expressed as the percentage of the total radioactivity released into the supernatant (*7*). Release of 80 percent of the isotope corresponds to 100 percent erythrocyte lysis (*7*, *9*).

Erythrocytes at concentrations of $<5 \times 10^5$ per incubation tube (= 1.5 ml) lost approximately 60 percent of their isotope within 24 hours. The same was the case when labeled lymphocytes were incubated at this low concentration. Increase of the serum concentration (fetal calf serum or fowl serum) in the medium from 5 to 30 percent had no effect. The spontaneous lysis of the erythrocytes was prevented when the cell concentration in the incubation tubes was increased 10 to 100 times, either by addition of viable lymphocytes, tissue culture cells, or even erythrocytes of various origins. Therefore, an appropriate excess of unlabeled fowl erythrocytes was added to the tubes instead of the lymphocytes in the lymphocyte-free controls. Under these conditions, the spontaneous release of isotope during 24 hours of incubation was usually less than 10 percent, and even after 40 hours it only rarely exceeded 20 percent. Lymphocytes incubated at concentrations of 1 to 10 $\times 10^6$ per incubation tube remained viable to 70 to 80 percent during this 40-hour period, as assessed by dye exclusion or isotope release. Presence or absence of PHA in the incubation mixture did not affect these figures.

Fowl erythrocytes incubated with lymphocytes in excess and with PHA present were significantly damaged. Independent duplicates almost never varied by more than 2 percent in regard to isotope release. Erythrocyte damage was not due to lysis of the lymphocytes (Table 1, experiments 1 and 2). The labeled lymphocytes were as active as unlabeled lymphocytes in bringing about a cytotoxic reaction. Addition of homogenized lymphocytes to normal viable lymphocytes had no toxic effects on the fowl erythrocytes (*6*, *7*). The active principle in PHA could be removed by previous absorption with lymphocytes (Table 1, experiment 3). This did not abolish the erythrocyte-agglutinating action of PHA. Heating it for 5 minutes at 100°C also extinguished its effect on lymphocyte cytotoxicity.

When lymphocytes were incubated with PHA for 1 hour before the fowl erythrocytes were added, cytotoxicity was actually stronger than when the cells were mixed before the addition of PHA (Table 1, experiment 4). Figure 1 shows the results of a typical experiment in which lymphocyte cytotoxicity was determined at different doses of PHA. In parallel experiments, the stimulatory effect of PHA on DNA synthesis (*1*) in the lymphocytes was also measured. Both curves had similar optima at 0.025 to 0.05 ml of PHA per

Table 1. Isotope release from labeled fowl erythrocytes or blood lymphocytes, respectively, incubated together for 42 hours in the presence or absence of PHA [0.025 ml of a stock solution of PHA per milliliter of incubation mixture (*7*)]. In experiment 1, the ratio of lymphocytes to erythrocytes was 50:1, while in the others it was 100:1. For labeling of the erythrocytes, 0.1 ml of a suspension (2 to 4 $\times 10^8$ cell/ml) in tris-buffered BSS containing 4 percent heat-inactivated fowl serum was incubated for 1 hour at 37°C with 100 μl (70 to 100 μc) sodium chromate-^{51}Cr [specific activity approximately 300 μc/μmole (*8*)]. The labeled cells were washed four times by centrifugation at 1000 rev/min and were finaly suspended in Parker 199. For labeling of the lymphocytes, 2×10^7 cell/ml were suspended in plasma. Samples (1 ml) were incubated with 100 μc ^{51}Cr-chromate for 1 hour at 37°C under gassing with 95 percent air and 5 percent CO_2. The cells were then washed three times in tris-buffered BSS and used as usual. In the lymphocyte-free control of experiment 1 (−), the lymphocytes were replaced by an equal number of unlabeled fowl erythrocytes. All numbers are the means from two duplicate incubations.

Erythrocytes	Lymphocytes	Isotope release (%)	
		PHA	No PHA
	Experiment 1		
+*	+	40	11
+*	−	11	14
+	+*	8	8
	Experiment 2		
+*	+	48	12
+	+*	17	22
+*	+*	30	14
	Experiment 3		
+*	+	52	21
+*	+	17†	
+*	+	14‡	
	Experiment 4		
+*	+§	63	
+*	+	53	8

* Labeled with ^{51}Cr. † PHA absorbed with lymphocytes for 2 hours. ‡ PHA absorbed with lymphocytes for 26 hours. § Lymphocytes preincubated with PHA for 60 minutes.

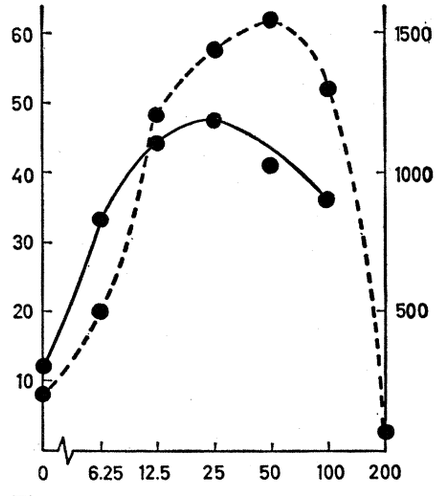

Fig. 1. Percentage of isotope release (solid line) from ^{51}Cr-labeled fowl erythrocytes (left ordinate) after their exposure to an excess (100:1) of fowl lymphocytes for 40 hours at different doses of PHA (abscissa). The doses are expressed as microliters of PHA stock solution per milliliter of incubation mixture (Table 1). The incubation medium was Parker 199 containing 5 percent heat-inactivated fetal calf serum. All points are means from duplicate incubations. *Dashed line:* radioactivity (count/min) in DNA of lymphocyte sample (right ordinate), incubated with ^{14}C-thymidine at the PHA doses indicated on the abscissa; 20×10^6 lymphocytes/ml were incubated for 40 hours, at which time 0.1 μc ^{14}C-thymidine (15 mc/mmole) was added. After incubation for an additional 88 hours, two 0.1-ml portions of each sample were extracted three times with ice-cold trichloroacetic acid (containing an excess of unlabeled thymidine), alcohol, alcohol-ether-chloroform, and finally ether. The radioactivity was determined in a liquid scintillation counter.

milliliter of incubation mixture. In contrast, mixed aggregation was strongest at the highest doses. Thus, cytotoxicity is correlated with the lymphocyte-stimulatory action of PHA rather than with its aggregating effect. It could then be argued that withdrawal of nucleotides from the nondividing but nucleated target cells by the stimulated lymphocytes is of major importance for this cytotoxic reaction. However, previous results with tissue culture cells and antimetabolite-treated human lymphocytes would seem to suggest that DNA synthesis and cytotoxicity are not causally related. Rather, they indicate that both may be independent expressions of lymphocyte stimulation (*3*, *6*).

For a clear-cut manifestation of cytotoxicity, an excess of lymphocytes over erythrocytes (at least 5:1) was required. Optimum effects usually appeared at ratios of lymphocytes to erythrocytes of 25:1 to 100:1. Under these conditions, significant cytotoxic effects were noted within a few hours, but 30 to 48 hours were usually necessary for complete hemolysis. Five percent of heat-inactivated fetal-calf serum and fowl serum both supported the reaction in the same way.

Table 2. Isotope release from ^{51}Cr-labeled fowl erythrocytes (Table 1). The cells were incubated with lymphocytes or other cell types of various origins at different ratios and for different times. All numbers are the means from two duplicate incubations. Abbreviations: RBC, erythrocytes; CLL, blood lymphocytes from patient with chronic lymphatic leukemia; BL, Burkitt lymphoma (freshly isolated cells from an African patient); F, fowl; H, human; the letters in parentheses indicate the blood group of individual from whom the cells were obtained; GP, guinea pig. Ly, lymphocytes.

Aggressor cells	Ratio to RBC	Isotope release (%)	
		PHA	No PHA
Experiment 1, 40 hours			
H-Ly (O)	50:1	38	3
Chang liver	20:1	9	9
Experiment 2, 22 hours			
H-Ly (A)	80:1	19	8
H-RBC	500:1	4	2
Experiment 3, 16 hours			
GP Spleen	50:1	29	8
F-RBC	50:1	11	
Experiment 4, 16 hours			
F-Ly	100:1	28	1
BL	7:1	7	10
BL	70:1	5	2
F-RBC	100:1	3	2
Experiment 5, 42 hours			
H-Ly	100:1	50	24
H-Ly	50:1	50	27
H-Ly	25:1	47	19
CLL	100:1	42	20
CLL	50:1	31	24
CLL	25:1	28	28
F-RBC	100:1	19	25

Cells from the peripheral lymphoid system are active in this system, regardless of their species origin (Table 2). This is also valid for rat and guinea pig blood lymphocytes. Tissue culture cells, erythrocytes of different species origin, and malignant lymphoid cells (human) such as Burkitt lymphoma cells (fresh or cultured) were completely inactive. Others, such as blood lymphocytes from patients with chronic lymphatic leukemia, occasionally exhibited cytotoxicity at elevated ratios of lymphocyte to erythrocyte, probably because of the presence of normal lymphocytes. Identical results were obtained with thymus cells from humans and guinea pigs; PHA caused strong mixed agglutination in all cases. The results suggest that the cells responsible for cytotoxicity are the PHA-responsive (probably the immunologically competent) lymphocytes (*6*). Erythrocyte phagocytosis was only exceptionally seen. However, since PHA-stimulated lymphocytes may acquire surface properties typical for macrophages, it is possible that some of the steps in phagocytosis and the cytotoxicity described here represent related phenomena.

Stimulation of human lymphocytes from Mantoux-positive individuals with PPD (purified protein derivative) makes them cytotoxic for immunologically unrelated tissue culture cells. Prior incubation of allogenic human lymphocytes in mixed culture also renders them cytotoxic for tissue culture cells (*3*). This indicates that the cell-killing reaction is independent of the stimulatory step in regard to immunological specificity. For this reason, and since it is not inhibited by inhibitors of protein and nucleic acid metabolism (*6*), cytotoxicity has not been assumed to be an expression of antibody synthesis by the aggressor cells. However, lymphocytes do not seem to kill target cells without discrimination. Tissue culture cells or tumor cells from rats or mice are killed by xenogenic, allogenic, or even semisyngenic rather than by syngenic lymphocytes (*4*, *5*). Similar observations hold for human skin fibroblasts treated with allogenic or autologous blood lymphocytes (*10*). This has led some authors to conclude that target-cell killing by lymphocytes is an expression of allogenic inhibition in which the target cells are killed by their exposure to foreign histocompatibility factors (*5*, *11*).

Although the data presented here and elsewhere (*6*, *7*) are incompatible with this concept, it is reasonable to ask whether histocompatibility differences between aggressor and target cells are absolute requirements for manifestation of the cytotoxic activity of stimulated lymphocytes from normal donors. Fowl erythrocytes, which, for instance, are known to carry histocompatibility factors (*12*), were lysed equally well by autologous and allogenic lymphocytes when PHA was present (Table 3). There were no differences over a wide range of ratios of lymphocytes to erythrocytes and over varying incubation periods. In contrast, human erythrocytes were not at all affected under these conditions, while sheep erythrocytes often showed a weak cytotoxic response to lymphocytes from fowl or other species (Table 3, experiment 5). Thus, hemolysis induced by PHA-treated lymphocytes seems to be independent of differences in histocompatibility between the cells. Whether hemolysis requires the presence of particular antigenic surface structures on the erythrocytes or is due to other properties of the cells is not known.

If the reactions described here are principally similar to those encountered with tissue culture targets—and our data strongly support this—we must conclude that a difference in histocompatibility between lymphocytes and target cells, although important in many

Table 3. Isotope release from ^{51}Cr-labeled erythrocytes (Table 1). The cells were incubated with autologous or allogenic fowl lymphocytes for different times and at different ratios. In experiment 5, labeled sheep or human erythrocytes (blood group A) were used as well. All numbers are the means from two duplicate incubations. RBC, erythrocytes; F, fowl; the numbers after F designate the origin of the cells from individual fowls.

RBC	Lymphocytes	Ratio to RBC	Isotope release (%)	
			PHA	No PHA
Experiment 1, 24 hours				
F-24	F-24	5:1	32	14
F-24	F-23	5:1	32	12
F-24	F-24	10:1	45	12
F-24	F-24	10:1	38	14
Experiment 2, 5 hours				
F-7	F-7	100:1	9	1
F-7	F-10	100:1	8	1
F-10	F-10	100:1	11	3
F-10	F-7	100:1	13	2
Experiment 3, 40 hours				
F-6	F-6	100:1	60	5
F-6	F-8	100:1	50	4
F-8	F-8	100:1	43	4
F-8	F-6	100:1	60	6
Experiment 4, 42 hours				
F-3	F-3	100:1	58	13
F-3	F-4	100:1	73	10
Experiment 5, 42 hours				
F-6	F-6	100:1	63	7
Sheep	F-6	100:1	20	4
Human (A)	F-6	100:1	14	8

situations, is not an absolute requirement for this cytotoxic reaction. This may be true for the lysis of tissue culture cells as well, since human lymphocytes often are cytotoxic for autologous skin fibroblasts, although this reaction may require higher lymphocyte concentrations and longer incubation periods than the allogenic combinations (*10*, *13*). However, the present findings do not indicate that the cytotoxic activity of stimulated lymphocytes is exerted without discrimination in regard to surface structures on the target cells. It cannot be excluded that a recognition step may occur somewhere in the chain of events. Although probably not bound to antibody secretion, this recognition could still be ascribed to receptor molecules on a fraction of the lymphocytes, in some way put into action by stimulation. The receptors could be lymphocyte-associated immunoglobulins or their subunits, with a specificity for target cell determinants. Reaction with the antigens or formation of antigen-immunoglobulin complexes could then trigger the injurious events in many different ways. In an alternative model, the degree of similarity between lymphocyte and target cell surface could determine the outcome of their interaction. In this model, recognition could, for instance, also be explained on the basis of quantitative differences in cell surface concentration or availability of histocompatibility factors. Although this concept would recognize the importance of histocompatibility factors, it would be different from that of allogenic inhibition in its present formulation (*11*), since it would allow for cytotoxic reactions in strictly autologous situations.

The role of lymphocyte cytotoxicity in the course of the various tissue-damaging immune responses mentioned above remains to be established. It would also be important to know how the present findings are related to such phenomena as the emission of macrophage inhibitory activity from sensitized lymphocytes upon contact with antigen, or to the release of leukotactic factors from allogenic lymphocytes kept in mixed culture (*14*). The simplicity of the test system described here should make it well suited for further studies of some of the problems outlined above.

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References and Notes

1. R. W. Dutton, *Advances Immunol.* **6**, 254 (1967); P. Perlmann and G. Holm, in *Mechanisms of Inflammation Induced by Immune Reactions*, P. Miescher, Ed. (Schwabe, Basel, Switzerland, 1968).
2. W. Rosenau and D. C. Morton, *J. Nat. Cancer Inst.* **36**, 825 (1966); I. Hellström and K. E. Hellström, *Science* **156**, 981 (1967); G. Holm, thesis (Balder, Stockholm, Sweden, 1967); D. W. Watson, A. Quigley, R. J. Bolt, *Gastroenterology* **51**, 985 (1966).
3. G. Holm and P. Perlmann, *J. Exp. Med.* **125**, 721 (1967)
4. G. Holm, P. Perlmann, B. Werner, *Nature* **203**, 841 (1964); G. Holm and P. Perlmann, *ibid.* **207**, 818 (1965).
5. E. Möller, *Science* **147**, 873 (1965); G. Möller and E. Möller, *Nature* **208**, 260 (1965).
6. G. Holm, P. Perlmann, B. Johansson, *Clin. Exp. Immunol.* **2**, 351 (1967); G. Holm, *Exp. Cell Res.* **48**, 327, 334 (1967); G. Holm, *Scand. J. Haematol.* **4**, 230 (1967).
7. G. Holm and P. Perlmann, *Immunology* **12**, 525 (1967).
8. Parker's medium 199 and antibiotics were obtained from Microbiological Associates, Bethesda, Md. Medium supplemented with PHA was from Difco Laboratories, Detroit, Mich. All isotopes used were from the Radiochemical Centre, Amersham, England.
9. H. S. Goodman, *Nature* **190**, 269 (1961); A. R. Sanderson, *ibid.* **204**, 250 (1964); H. Wigzell, *Transplantation* **3**, 423 (1965).
10. G. Möller, V. Beckman, G. Lundgren, *Nature* **212**, 1203 (1966).
11. I. Hellström and K. E. Hellström, *Ann. N.Y. Acad. Sci* **129**, 724 (1966); K. E. Hellström and G. Möller, *Progr. Allergy* **9**, 158 (1965).
12. D. G. Gilmour, *Ann. N.Y. Acad. Sci* **97**, 166 (1962); L. W. Schierman and A. W. Nordskog, *ibid.* **120**, 348 (1964).
13. E. Chu, J. Stjernswärd, P. Clifford, G. Klein, *J. Nat. Cancer Inst.* **39**, 595 (1967).
14. B. R. Bloom and B. Bennett, *Science* **153**, 80 (1966); J. R. David, *Proc. Nat. Acad. Sci. U.S.* **56**, 72 (1966); H. Ramseier, *Science* **157**, 554 (1967).
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Gas Chromatography for Detection of Viral Infections

Abstract. *Gas chromatograms of serum extracts of dogs inoculated with canine infectious hepatitis virus showed two metabolites not observed in uninoculated animals. Chromatograms of extracts of tissue cultures of dog kidney, inoculated with viruses causing canine hepatitis, herpes, and distemper, and a parainfluenza virus similar to simian virus-5, each showed two or more different metabolites. Two of the distinguishing products from cultures inoculated with hepatitis virus were chromatographically indistinguishable from those found in serums of the animals.*

Recent evidence suggests that gas-chromatographic techniques may provide the basis for a new methodology for the rapid detection and differentiation of free-living microorganisms grown in laboratory media. Distinctive chromatographic patterns have been observed for each of the bacteria examined by procedures designed to detect excreted metabolites that are volatile or can be made volatile, and the products of less than a single bacterial cell may be sensed by chromatographs having suitable detectors (*1*). Distinctive gas-chromatographic signatures can also be obtained when certain cell constituents are examined directly (*2*) or after pyrolysis (*3*). In view of the marked sensitivity of the electron-capture detector to certain metabolites (*4*), we tried to determine whether products appearing in low concentrations as a result of infection could be observed, and whether different pathogenic agents would yield different metabolites.

Healthy mixed-breed dogs, shown by virus-neutralization tests to be susceptible to infectious canine hepatitis virus, were kept in isolation units. After 2 to 4 weeks of observation the animals were inoculated with 1.0 ml of a virus suspension containing $10^{6.3}$ $TCID_{50}$ (tissue-culture infectious dose affecting 50 percent). Dogs were bled for serum prior to inoculation with the virus, and daily or occasionally less frequently thereafter until the animals either died or recovered. Serums were collected from clotted blood within 2 hours of bleeding and stored at −60°C.

The thawed samples of serum (2.0 ml) were treated with 0.2 ml of 5*N* HCl and 1.0 ml of 0.2*M* HCl–KCl buffer, *p*H 2.0, and dried under vacuum; they were then dissolved in pyridine and treated with hexamethyldisilazane and trimethylchlorosilane. A 3-μl portion of the supernatant was injected into an Aerograph model-200 gas chromatograph fitted with electron-capture and flame-ionization detectors. The stainless steel column, 1.83 m long and 3 mm in diameter, was packed with Chromosorb-W treated with hexamethyldisilazane. The liquid phase was 10 percent Carbowax (20-M), and the operating temperatures were 100°, 120°, and 150°C for the column, detectors, and injector, respectively. Nitrogen was the carrier gas.

Serum extracts from all 11 experimental animals contained constituents having retention times (R_t) of about 20, 40, 60, 110, 140, and 190 seconds