antibody preparations studied, most, if not all, of the peptides observed come from the invariable portions of the L chains and Fd' fragments. The results obtained are, therefore, not surprising, but beforehand it was considered possible that if the "PLL gene," which is required for the immune response to DNP-PLL codes for some partial specificity for PLL, it could determine the sequence of a common segment in the variable portion of the L chain or the Fd fragment of anti-DNP-PLL antibodies produced by responder guinea pigs. Such a segment would be expected to be absent in antibodies produced by guinea pigs lacking the PLL gene. If such a structural difference did exist, it might result in extra peptides in the maps of antibodies from genetic responder animals. This result was not observed. The minor differences consisting of 3 faint spots present only in the maps of $F(ab')_2$ fragments from responder animals would seem to be insufficient to support this interpretation for the following reasons: the 3 spots in question were relatively faint and probably do not represent sequences common to all the molecules in the sample, and in other experiments similar spots were observed in maps of $F(ab')_2$ fragments from normal nonspecific immunoglobulins and from anti-DNP-bovine gamma globulin (BGG) antibodies (Fig. 1 D). Therefore, these experiments are consistent with the concept that the PLL gene does not control directly the strucure of anti-hapten-PLL antibodies but acts at an earlier step in the immune response, perhaps at the stage where antigen has to be specifically processed to be immunogenic.

Summary. Peptide map of $F(ab')_2$ fragments of anti-DNP-PLL antibodies obtained from responder guinea pigs immunized with DNP-PLL and from guinea pigs lacking the PLL gene immunized with DNP-PLL • ovalbumin complexes were compared. No structural differences that could be attributed to the "PLL gene" were observed.

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1. Levine, B. B., Ojeda, A., and Benacerraf, B., J. Exptl. Med. **118**, 953 (1963).

2. Levine, B. B. and Benacerraf, B., Science **147**, 517 (1964).

3. Green, I., Paul, W. E., and Benacerraf, B., J. Exptl. Med., **123**, 859 (1966).

4. Green, I., Vassalli, P., and Benacerraf, B., J. Exptl. Med. **125**, 527 (1967).

5. Farah, F. S., Kern, M., and Eisen, H. N., J. Exptl. Med. **112**, 1195 (1960).

6. Nisonoff, A., Methods Med. Res. **10**, 134 (1964).

7. Small, P. A., Jr. and Lamm, M. E., Biochemistry **5**, 259 (1966).

8. Katz, A. M., Dreyer, W. J., and Anfinsen, C. B., J. Biol. Chem. **234**, 2897 (1959).

9. Lamm, M. E., Lisowska-Bernstein, B., and Nussenzweig, V., Biochemistry **6**, 2819 (1967).

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Biologic Activity of African Lymphoma Extracts* (32893)

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The present report describes certain biologic effects observed in cultured cells following treatment with extracts of malignant lymphomas (Burkitt's tumor) collected in Africa. The observations were made as part of a combined field (1) and laboratory study of the disease which is a common form of malignancy among children in central Africa (2) and New Guinea (3) and occurs infrequently elsewhere.

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Materials and Methods. Patients. The illnesses represented in the present study conformed to the pattern described by workers in Africa (4, 5) and the average age of the patients was seven years.

Specimens. Tumor biopsies were collected in the operating room where cytologic preparations were made and samples selected for histologic examination. Since many of these tumors contain large areas of necrosis, the

samples were usually dissected and only the viable areas chosen for culture. Bone marrow samples were collected from as many sites as possible and usually pooled. Tumor, bone marrow, and fecal samples were promptly frozen and stored with dry ice. Fresh blood samples were allowed to coagulate at room temperature and the serum and clot were frozen separately.

Eight solid tumors of mice (melanomas and Ehrlich carcinomas) and 4 spleens of leukemic mice (Friend virus leukemia and Rauscher virus leukemia) were tested immediately after autopsy and extract preparation.

Ten tumor (lymphoma) cell cultures were tested. Eight of these (EB-1, EB-2, EB-3, Kudi, Jiyoye, Raji, Ogun, and SL-1) were provided by Dr. L. Old. The B-25-M was given to us by Dr. G. Moore. The Makoha cell line was established in this laboratory.

Tissue culture. Tumor and fecal specimens were ground with alundum, suspended in saline, centrifuged lightly and the supernatants were applied to cell cultures of primary human amnion (PHA), human embryonic kidney (HEK) and human embryonic lung (WI-26). The marrow and blood clot specimens were homogenized, centrifuged, and supernatants were inoculated into the same three cell cultures.

The PHA cultures were maintained in various media: Bodian's, LY, M150 or Eagle's basal medium. The HEK cultures were maintained in monkey kidney medium B. WI-26 cells were carried in LY medium. The media were all supplemented with fetal calf serum, final concentration 5%, and all contained 100 units of penicillin and 100 μg of streptomycin/ml.

Blind passages of tissue culture fluids were made into primary cell cultures and into additional cell lines including human amnion (FL), hamster lung (Hsu), grivet monkey kidney (GMK), baby hamster kidney (BHK-21) and human embryonic lung (HEL). FL, BHK-21 and HEL cells were maintained in Eagle's basal medium with 5% fetal calf serum; Hsu cells in Eagle's minimal essential medium with 5% fetal calf serum; GMK cells in Monkey Kidney Medium B with 2% fetal calf serum.

In addition to passing supernatant fluids from inoculated cultures, passages of intact cells and of extracts of disrupted cells were made. Cells were either trypsinized or scraped gently from the growing surface and seeded into new cell cultures or into new containers. Some cultures were rapidly frozen and thawed three times, centrifuged, and the supernatants were inoculated into fresh cell cultures. Others were ultrasonicated, centrifuged, and the supernatants were passed.

Challenge of amnion cultures. For challenge, the following viral suspensions were used: Sindbis virus strain 167H chicken fibroblast passage; Bunyamwera virus strain 49 mouse passage, Hsu passage; Echo virus type 11 GMK passage, GMK passage.[1] Fresh tissue culture harvests of the viruses were centrifuged at 3000 rpm for 15 min and the supernatants stored at —80°C. Titrations were performed in PHA tube cultures, and 100 and 1000 $TCID_{50}$ were used for challenge. Cell cultures were washed twice with maintenance medium, inoculated with the virus suspension and re-fed with maintenance medium.

Interferon assay. Supernatant fluids of altered amnion cultures and of uninoculated controls were withdrawn and centrifuged at 1000 rpm for 15 min. A 0.2-ml portion of the supernatants was inoculated into each of 4 or 6 tube cultures of PHA. Twenty-four hours later, 100 $TCID_{50}$ of challenge virus were added to half of these tubes.

Actinomycin D treatment. Four specimens causing the PHA alterations were tested again in PHA cultures pretreated for 4 hours with Actinomycin D1, according to the method of Heller (6).

Results. Amnion alterations. Thirty-nine specimens, from 18 patients, were tested in PHA cell cultures as described, and 13 induced irreversible morphological changes. The frequency of activity was higher with tumor extracts (5 active of 10 tested) than with bone marrow (5 active of 15 tested), and lowest with blood clots (3 active of 14 tested).

The morphological changes usually appeared between the sixth to the eighth day

[1] The Sindbis virus was provided by John Enders, the Bunyamwera by Dr. R. Speir and the Echo strain by Dr. H. Malherbe.

after inoculation. Most cultures had by then had 2 or 3 fluid changes. The cells became elongated and curved, and aligned to form twisting patterns in a luxuriant hyaline sheet with smooth edges. Such cells appeared less granular, less vacuolated, smoother and more three-dimensional than the normal polygonal, flat PHA cells. They were also very different from the thin, sharp fibroblastic cells sometimes appearing in old cultures. The entire culture was usually involved within the day, and altered sheets endured for many weeks, long after the control cultures showed complete degeneration. No loss of contact inhibition occurred, and no pH changes appeared.

Stained altered cultures often showed a linear array of 3 or 4 nuclei in an intensely eosinophilic cytoplasm, the cells arranged in tightly-packed whorls interspersed with a few flat polygonal cells (Figs. 1 and 2). No increase in mitotic activity was observed.

The active specimens repeatedly induced the alterations, but attempts to transfer the effect to new PHA cultures were fruitless. Supernatant fluids from altered cultures were passed directly to new cells, or after fractionation by centrifugation. When volume permitted, transfers of intact altered cells were made, or extracts of disrupted altered cultures were passed. The morphologic effect was never transferred.

When the size of tumor samples permitted, extracts were prepared from 20% suspensions of tissue. In these instances the titer of the metamorphic activity was tested using serial dilutions of the extract. In only one instance did a 1 to 10 dilution induce the morphologic alterations. In all other cases, only the undiluted fluids were active.

The inoculation of PHA cultures with two specimens that had caused metamorphosis was repeated incorporating patients' sera into the maintenance medium. Two sera, one from a patient in remission and the other from a patient in an early stage of the disease, were substituted for the usual serum supplement in the tissue culture medium. These cultures were maintained as before, except always in the presence of the patients' sera. The cell alterations occurred as before, unaffected by the presence of the sera.

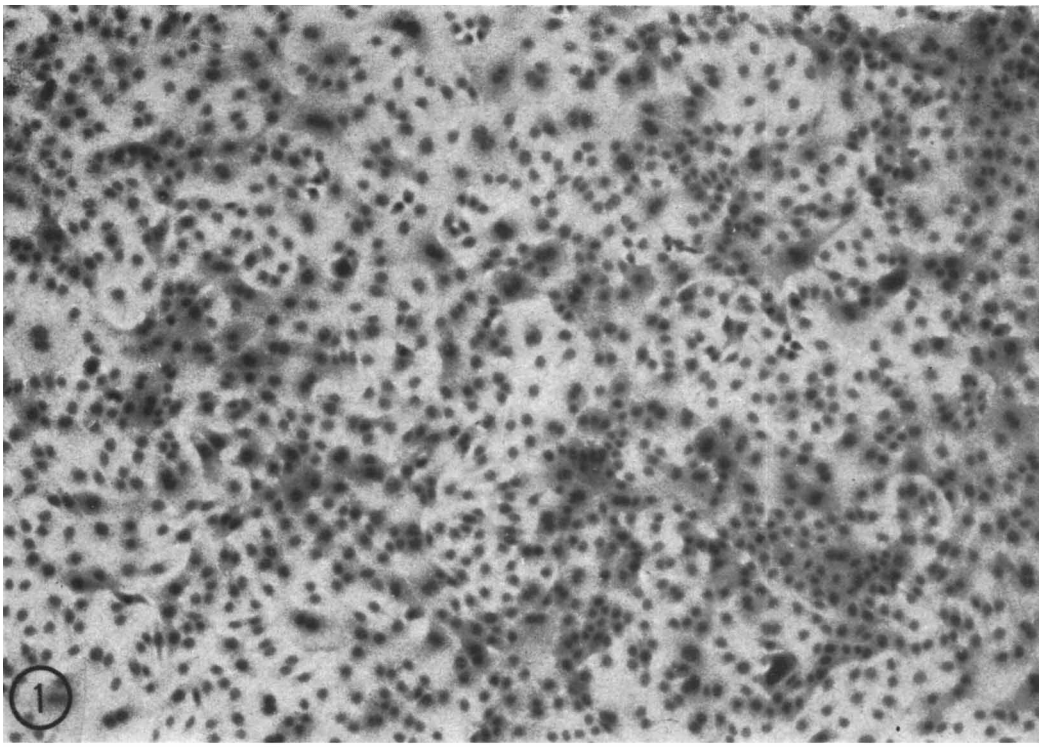
Thirty-seven specimens, mostly bone marrows and blood clots, from 26 patients with other neoplastic diseases, but including 11 with leukemia and 1 with Hodgkin's disease, failed to induce PHA changes. Stool specimens from the same patients were also tested. Two of these induced morphologic alterations of PHA cells but in both cases late CPE occurred and adenoviruses were isolated.

Similarly tested in PHA cultures were freshly prepared extracts of 8 solid tumors of mice and 4 spleens of leukemic mice. None induced morphological or other visible alterations of the cells. The ten established Burkitt tumor cell lines also failed to induce amnion lesions. Seven of these have been reported by others to contain herpes-like virus particles.

A degree of cellular proliferation and realignment suggestive of the initial stage of alteration was observed in PHA cultures during the early stages of infection with a poxvirus isolated from one of the African lymphomas. Between foci of CPE, areas of increased cell density appeared with a luxuriant, hyaline aspect and realignment of cells resembling the changes associated with active extracts. However, in this case the cells did not undergo the elongation and curving characteristic of the other activity and the effect disappeared within a few days as the CPE spread throughout the culture. In repeated tests of this tumor and the poxvirus isolate with varying dosage and inactivation methods, the temporary proliferative response often appeared but permanent alteration was never produced.

Mycoplasma had been isolated from a number of the specimens (7) and representative strains were tested for their effect on amnion cell cultures. None induced the characteristic changes. Massive doses (10^6 colony forming units/ml) commonly caused a faintly granular appearance and inconstantly a degree of gradual necrosis. Lower doses (10^3–10^5 CFU/ml) were followed by more rapid growth of the amnion cells and an increase in the mitotic index during the 5 days following inoculation.

Thirty-nine specimens that were comprehensively tested both for the amnion effect and the presence of mycoplasma (Table I)



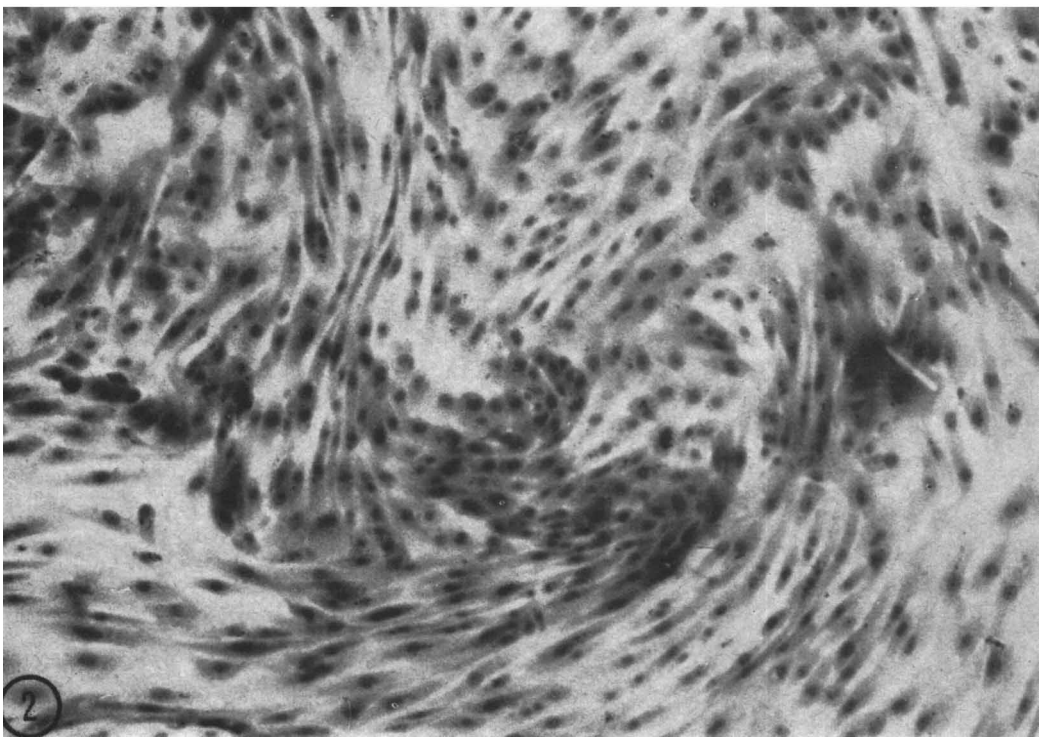


FIG. 1. Uninoculated primary human amnion culture, H. and E, 100 ×.
FIG. 2. Morphologic responses of amnion cells to a tumor extract; day 18; H and E, 100 ×.

TABLE I. Results of Comprehensive Tests for the Presence of Mycoplasma and the Amnion Effect.

Specimen	No. tested	The no. showing			
		Amnion effect only	PPLO only	Amnion effect and PPLO	Neither
Tumor	10	4	1	1	4
Bone marrow	15	4	3	1	7
Blood clot	14	2	1	1	10

showed that the two were only erratically associated. Nor did kanamycin prevent the evolution of the morphologic response when the amnion cultures were kept in presence of 200 μg/ml of kanamycin, a dose greatly in excess of the minimal mycoplasma-inhibiting dose (10 μg/ml).

Resistance to viral infection. The persistence of the PHA effect distinguished it from similar changes others have associated with the action of interferon (8). Moreover, supernatants of the altered cultures gave no evidence of interferon activity. Nor did pretreatment of the cells with Actinomycin D influence the outcome. Nevertheless altered PHA cultures when challenged with 100 to 1000 $TCID_{50}$ of Sindbis or Bunyamwera viruses were completely resistant as judged by the absence of cytopathic changes. Several altered cultures similarly resisted infection by an Echo virus (type 11).

Isolation of viruses and other agents. Viruses were at times isolated from lymphoma specimens. The results have been reported elsewhere (9). Six viruses were isolated directly in PHA. One enterovirus strain, untyped, was isolated from a tumor extract. The vaccinia virus was recovered from a tumor which had been sampled 11 days after vaccination. Four viruses were recovered from fecal specimens: 2 reoviruses, 1 adenovirus, and 1 Coxsackie B virus. Candida albicans was recovered twice from tumors of the oral cavity.

Discussion. We have no direct evidence of relationship of the amnion lesions to the disease other than the frequent association (13 of 39 specimens induced the changes) and the greater frequency of activity in tumor specimens than in marrow or blood clot. That bone marrow cells showed activity is consonant with our conception of these African tumors as a form of malignant lymphoma of childhood that is conditioned by environmental forces (10). Patients included in the present series frequently were demonstrated to have bone marrow lesions compatible with lymphoblastic leukemia.

Somewhat similar changes of primary human amnion cultures have been attributed by others to interferon (8), to infection with a variant type 2 poliovirus (11) and to treatment with human neoplastic specimens (12). Rather similar changes in primary amnion cells were encountered by Bernkopf *et al.* (13) following inoculation with inactivated vaccinia virus. However in this instance the lesions appeared within a day after exposure of the cells and led to disintegration shortly afterward. They suspected toxic activity.

The time relationships we have met resemble the effects of defective viruses encountered by others in the search for tumor viruses (14). Interestingly we have observed the amnion alterations in cultures which eventually yielded known viruses (several adenoviruses, one enterovirus and two reoviruses). In these cases the morphologic changes preceded frank cytopathogenicity by considerable periods and since the isolates were incapable of reproducing the morphologic effect it may be questioned whether two agents played a part.

There is reason to believe that the amnion response may be governed by factors extrinsic to the inocula since specimens tested earlier in amnion cell cultures in the presence of horse rather than fetal calf serum yielded no such effects. Influence of serum type on morphologic alterations of cells has been noted by others (14–17).

Nucleic acid preparations are said to induce morphologic changes in cultured cells. Jones'

observations of cell alterations caused by tumor RNA (18) and the report by Shepley *et al.* of permanent alterations induced by nucleic acids (19) may be relevant. Of particular interest is the report of Osato and Ito of morphologic alterations in human embryonic cells following treatment with cell-free supernatants of cultured human leukemic cells (20). It was not stated whether such altered cells were resistant to infection.

Resistance to superinfection has been characteristic of our experience and seems to imply the presence of infection and interference either by intracellular competition or by interferon production. It is tempting to think of the latter because of the resemblance of the changes to the interferon effect in primary human amnion cells described by Gresser (8). Others have reported interferon production and resistance to viral infection by cell lines derived from Burkitt lymphoma (21–23). Uncertainty on this and other aspects of our study is a consequence of the limited size of the specimens and the failure to devise a continuous source of the activity.

We have no indication to associate the herpes-like virus frequently demonstrated in cultured Burkitt tumor cells with the amnion lesions. Various altered amnion cultures were examined visually (EM) without evidence of the presence of such viruses and none of the seven strains of cultured Burkitt tumor cells known to harbor the herpes-like forms (24) induced alterations in our amnion cultures.

The significance of these viruses which Epstein first found in cultured tumor cells and which have since, on one occasion, been seen in tumor as well (25) is a matter of lively interest. They have been transmitted to other cells (26, 27) but only under very special circumstances. Their association with the disease is supported by a number of serologic studies that seem to relate them to the antibodies sometimes present in children with lymphomas (28–31). On the other hand antibodies are more frequently present in the sera of patients with postnasal space cancers both in Africa and in North America (30). This has been true of our own serum specimens as well.

In Kenya where both postnasal space cancers and the jaw and ovarian lymphomas are relatively common we found the former largely limited to the higher and colder areas and the lymphomas to the lowlands. Thus the distribution of the herpes-like virus, as judged by serologic tests, does not conform to the geographic distribution of the lymphomas which is thought to be a characteristic of Burkitt's tumor. It is therefore difficult to relate the herpes-like virus with the original theory (32) of an infectious (virus) disease limited to areas where tropical mosquitoes are common and possibly mosquito-transmitted. If, on the other hand, the disease is a modified form of malignant lymphoma Epstein's virus may be the ubiquitous, primary etiologic agent. Alternatively it may be a widely distributed virus that has previously escaped detection because of its lack of pathogenicity for laboratory animals, difficulty of cultivation *in vitro* and lack of pathogenicity for man.

Summary. Extracts of African lymphomas (Burkitt's tumor) frequently induced morphologic alterations in cultured primary human amnion cells. The changes were associated with resistance to infection by several viruses.

June Biedler took part in these studies both in chromosomal studies and in mycoplasma testing. Jørgen Fogh was helpful in many ways and Elizabeth Hazen was responsible for mycologic examinations. Isobel Spence collaborated in serological and cultural studies. Theresa Gouaux was largely responsible for the mycoplasma testing.

1. Dalldorf, G., Linsell, C., Barnhart, F., and Martyn, R., Perspectives Biol. Med. **7**, 435 (1964).

2. Burkitt, D., E. African Med. J. **38**, 511 (1961).

3. Ten Seldam, R., Cooke, R., and Atkinson, L., Cancer **19**, 437 (1966).

4. Burkitt, D. and O'Conor, G., Cancer **14**, 258 (1961).

5. O'Conor, G., Cancer **14**, 270 (1961).

6. Heller, E., Virology **21**, 652 (1963).

7. Dalldorf, G. and Bergamini, F., Proc. Natl. Acad. Sci. U. S. **51**, 263 (1964).

8. Gresser, I., Proc. Natl. Acad. Sci. U. S. **47**, 1817 (1961).

9. Dalldorf, G., Bergamini, F., and Frost, P., Proc. Natl. Acad. Sci. U. S. **55**, 297 (1966).

10. Dalldorf, G., J. Am. Med. Assoc. **181**, 1026 (1962).

11. Ho, M., Proc. Natl. Acad. Sci. U. S. **46**, 1623 (1960).

12. Stewart, S. and Irwin, M., Cancer Res. **20**, 766 (1960).

13. Bernkopf, H., Nishmi, M., and Rosin, A., J. Immunol. **83**, 635 (1959).

14. Hartley, J. and Rowe, W., Proc. Natl. Acad. Sci. U. S. **55**, 780 (1966).

15. Rubin, H., Virology **12**, 14 (1960).

16. Svoboda, J., Intern. Rev. Exptl. Pathol. **5**, 25 (1966).

17. Evans, V., Jackson, J., Andresen, W., and Mitchell, J., J. Natl. Cancer Inst. **38**, 761 (1967).

18. Jones, P., Nature **202**, 1226 (1964).

19. Shepley, K., Ambrose, E., and Kirby, K., Nature **208**, 1072 (1965).

20. Osato, T. and Ito, Y., Proc. Natl. Acad. Sci. U. S. **57**, 1076 (1967).

21. Henle, G. and Henle, W., J. Bacteriol. **89**, 252 (1965).

22. Rabson, A., O'Conor, G., and Baron, S., Int. J. Cancer **1**, 89 (1966).

23. Epstein, M., Achong, B., Barr, Y., Zajac, B., Henle, G., Henle, W., J. Natl. Cancer Inst. **37**, 547 (1966).

24. Henle, G. and Henle, W., Trans. N. Y. Acad. Sci. **29**, 71 (1966).

25. Manube, G., report to E. African Med. Res. Council, Nairobi, Kenya, Jan. 13, 1967.

26. Mitchell, J., Anderson, G., Bowles, C., and Hinz, R., Lancet **2**, 1358 (1967).

27. Henle, W., Diehl, V., Kohn, G., Hausen, H., and Henle, G., Science **157**, 1064 (1967).

28. Henle, G. and Henle, W., J. Bacteriol. **91**, 1248 (1966).

29. Henle W., Hummeler, K., and Henle, G. **92**, 269 (1966).

30. Old, L., Boyse, E., Oettgen, H., de Harven, E., Geering, G., Williamson, B., Clifford, P., Proc. Natl. Acad. Sci. **56**, 1699 (1966).

31. Gerber, P. and Birch, S., Proc. Natl. Acad. Sci. U. S. **58**, 478 (1967).

32. Haddow, A. and McCallum, D., Ann. Rept. E. African Virus Res. Inst., 1962.

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The Effect of Oral and Intravenous D-Ribose on Plasma Insulin Levels in Unanesthetized Dogs* (32894)

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Although it is widely acknowledged (1–6) that increased blood glucose levels have a direct stimulating effect on pancreatic β-cells, there has been evidence indicating that secondary, extrapancreatic mechanism(s) for insulin secretion exist. Some of this evidence has indicated that the response to D-ribose may involve such a mechanism. It has been shown that intravenous administration of ribose causes hypoglycemia in both man (7) and anesthetized dogs (3). In addition, intraportal infusion of ribose into anesthetized dogs has resulted in immunoassayable insulin secretion (8) although no insulin secretion was elicited from sliced rabbit pancreas incubated *in vitro* with a ribose-containing medium (6, 9). Since elevated epinephrine levels inhibit insulin secretion despite resulting hyperglycemia (10), it has been suggested that the increased insulin levels observed following intravenous sugar infusion represent, in part, reversal of epinephrine block of the β-cells in a surgically stressed animal (11). It was the purpose of this investigation to clarify further the above questions by studying the effects of oral and intravenous ribose on plasma insulin levels in awake, alert, unstressed dogs.

Procedure. Nine mongrel dogs ranging in weight from 12–22 kg were anesthetized with sodium pentabarbital and prepared for the experimental procedure. Silastic cannulas were placed in the femoral artery for future sampling and in one or two major veins (portal, femoral, or external jugular) for infusing D-ribose. After a minimum recovery period of 72 hours, the dogs were subjected to an 18-hour fast followed by an experiment. A minimum of 48 hours was allowed to elapse between two experiments on a given dog. In

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