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Bioautographic Analysis of Blood for Growth Factors* Active for *L. leichmannii* 4797. (19827)

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Previous observations by Couch *et al.*(1) on vit. B_{12} content of blood from various species have shown that the vit. B_{12} activity of turtle, chicken and turkey blood is higher than that of other species with the exception of the rabbit. These workers reported further that autoclaving blood samples from the chick, turkey and turtle at pH 12 destroyed only a part of the *L. leichmannii* activity. Such activity was explained on the basis of the fact that the latter species have nucleated erythrocytes and that the *L. leichmannii* response of the alkali treated and autoclaved blood samples was due to thymidine. A later study (2) reported values on vit. B_{12} content of blood from various species which were within the range of those reported by Couch *et al.*(1). The B_{12} content of blood from different breeds of cows has been determined in this laboratory(3). Blood samples from the Jersey breed contained the least B_{12} while those from Guernsey, Holstein and Brahman breeds were significantly higher.

The present study was designed to determine whether blood from species with nucleated erythrocytes contained growth factors for *L. leichmannii* 4797 other than vit. B_{12}.

Experimental. Five hundred ml of whole blood was collected from the cow, sheep and pig in oxalated containers. Blood samples from the chick, turkey and turtle were each pooled in order to obtain 500 ml of whole blood. In all cases blood samples were diluted with an equal volume of water, layered with toluene and incubated at 37°C for 24 hours. The autolysates were adjusted to pH 7 and autoclaved for 5 minutes at 120°C. After cooling, the samples were homogenized, centrifuged and the centrifugates were concentrated *in vacuo* to 20 ml. The vit. B_{12} content of each autolyzed blood sample (prior to concentration *in vacuo*) was determined according to the titrimetric method of Skeggs *et al.*(4) with *Lactobacillus leichmannii* 4797 as the test organism and crystalline vit. B_{12} as the standard. A 10 ml portion of each of the

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autolyzed blood concentrates (prepared as described above) from the chick, turkey and turtle blood were adjusted to pH 12 and autoclaved for 1 hour at 120°C. After cooling the samples were readjusted to pH 7 and concentrated *in vacuo* to a volume of 10 ml. Other blood samples were obtained by pooling 500 ml samples of fresh whole blood from the chick, turkey and turtle separately, mixing with an equal volume of water and autoclaving immediately for 15 minutes at 120°C. After cooling, the samples were layered with toluene, incubated at 37°C for 24 hours, reautoclaved at 120°C for 5 minutes. After cooling the samples were homogenized, centrifuged and the centrifugate concentrated *in vacuo* to a volume of 20 ml. The presence of *desoxyribonucleases and nucleotidases* in fresh chick blood was shown by incubation of sodium desoxyribonucleate (Nutritional Biochemical Corp.) solution (10% in dilute alkali) with the chick blood, chromatographing and bioautographing. Descending chromatography according to the procedure of Winsten and Eigen(5) was used to separate the growth factors active for *Lactobacillus leichmannii* 4797. Ten to 15 μl of each of the blood concentrates, DNA preparations, liver extract, vit. B_{12} (100 mγ/ml) or thymidine (50 γ/ml) were spotted on ½ x 16 inch paper strips (Eaton Dikeman No. 613). Water saturated with n-butanol was used as the developing agent. After removal from the chromatographic jars, the strips were air dried and bioautographed according to the method of Winsten and Eigen(6). In order to facilitate greater diffusion of vit. B_{12}, paper strips were allowed to remain on the surface of the seeded agar during the 16-hour period of incubation.

Results and discussion. Autolyzed blood samples from the chick, turkey, turtle, sheep, pig and cow contained 6.0, 5.2, 5.8, 0.8, 1.4, and 1.4 mγ of vit. B_{12} activity per ml of blood respectively as determined with *L. leichmannii*. Such values are within the range of those reported by earlier workers(1,2).

Tracings of bioautographs of liver extracts, concentrates of autolyzed blood samples from the chick, turkey and turtle, crystalline vit. B_{12} and thymidine are illustrated in Fig. 1.

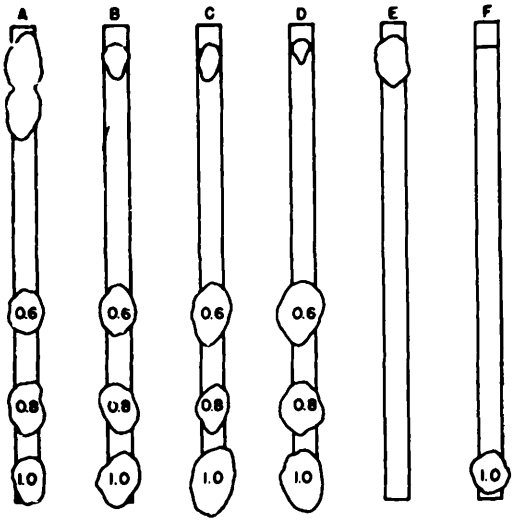

FIG. 1. Tracing of bioautographs. Relative rates* of active zones are given within the zone area. Strip A, 10 μl, 1:10 dilution of 15 U.S.P. unit liver extract (Lederle); Strip B, C, and D, 15 μl concentrates from autolyzed blood samples of chick, turkey, and turtle, respectively; Strip E, 10 μl sol. of crystalline vit. B_2 (100 mγ/ml); Strip F, 10 μl of thymidine sol. (50 γ/ml).

* Relative rate: (Distance from starting place to center of growth zone in question)/(Distance from starting place to center of growth zone for the fastest moving component)(7).

Liver extract solution (Strip A, Fig. 1) contains a stationary connected doublet zone (a part of which moved rather slowly) in addition to 3 more rapidly moving growth factors (6,7). Blood concentrates from chick, turkey and turtle (Strip B, C and D) each contained a growth factor for *L. leichmannii* 4797 which remained stationary as did vit. B_{12} (Strip E). Each of the concentrates also contained 3 additional growth factors which moved at the same relative rates (0.6, 0.8, and 1.0, Fig. 1) as did the 3 faster moving components of liver extract solution (Strip A, Fig. 1). The fastest moving growth factor in the liver extract and in 3 blood concentrates moved at the same relative rate as did thymidine (Strip F, Fig. 1). The growth factor in liver extract with the relative rate of 0.6 has been identified as hypoxanthine desoxyriboside(8). Blood concentrates from the chick, turkey, and turtle were also found to contain this component which moved at a relative rate of 0.6. An unidentified growth factor that moved with a relative rate of 0.8 was found to be present

in liver extract and in these 3 blood concentrates. These data show that blood samples from species with nucleated erythrocytes contain, in addition to vit. B_{12}, 3 growth factors for *L. leichmannii* 4797. Such factors increase the apparent vit. B_{12} activity of these blood samples when assayed by a standard microbiological assay procedure with *L. leichmannii* as the test organism.

Blood samples from the cow, sheep and pig contain only one growth factor, which occupied the same stationary position on a bioautograph as did B_{12}. Attention is directed to the fact that blood from cow, sheep and pig contained only one growth factor for *L. leichmannii* 4797 while that from chick, turkey and turtle contained 4 growth factors for *L. leichmannii* (Fig. 1) even though the blood samples from all species were treated in an identical manner.

Alkali treatment of blood concentrates from the chick, turkey, and turtle destroyed the growth factor which occupied the same stationary position on a bioautograph as did vit. B_{12}. It has been shown that vit. B_{12} is destroyed by autoclaving in an alkaline medium (9, 10). Couch *et al.*(1) showed earlier that the *L. leichmannii* activity of blood samples from species with nucleated erythrocytes was decreased by autoclaving at pH 12 for 1/2 hour. Autoclaving blood samples from the chick, turkey and turtle in an alkaline medium did not change the relative position of the 3 faster moving growth factors on a bioautograph, which shows that these components are stable to such treatment.

Autoclaved fresh blood samples from the chick, turkey and turtle contained only one stationary growth factor which occupied the same relative position on a bioautograph as did vit. B_{12}. It is significant that the 3 faster moving components shown on Fig. 1 did not appear when the samples of chick, turkey and turtle blood were autoclaved immediately after collection. Autoclaving of fresh blood samples apparently destroyed blood enzymes (desoxyribonucleases, and nucleotidases), which were required for the release of the desoxyribosides from the desoxyribonucleic acids.

Chick blood solution or DNA solution when incubated alone were devoid of growth factors. Incubation of DNA solution with chick blood resulted in the appearance of the 3 faster moving growth factors. The fastest moving component moved at the same relative rate as did thymidine. It is obvious from these data that enzymes present in chick blood released 3 growth factors active for *L. leichmannii* 4797 from DNA.

Summary. Aqueous extracts of autolyzed blood samples from various species were chromatographed on paper strips and analyzed by bioautographic procedures. Under these conditions chick, turkey and turtle blood were found to contain 3 desoxyribosides in addition to vit. B_{12} while cattle, sheep and pig blood contained only B_{12} but not the desoxyribosides. Evidence is presented for the occurrence of desoxyribonuclease and nucleotidase in chick blood.

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