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The Sedimentation Characteristics of Deoxyribonucleic Acid from Normal and Diseased Human Tissues

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Clinical investigations of certain anti-metabolites which interfere with metabolism of nucleic acid have shown that they act in different ways upon the leucocytes present in the bloodstream in myeloid and lymphatic leukaemia. This suggests that the nucleic acids of these leucocytes may differ. The physical properties and chemical composition of the deoxyribonucleic acid from

normal and leukaemic human leucocytes have been investigated by Polli (1957) and Polli & Semenza (1955, 1956). It was found that the effect of urea and of alkali on the viscosity of solutions of deoxyribonucleic acid prepared from myeloid leukaemia leucocytes was different from that of solutions of deoxyribonucleic acid from normal or lymphatic leukaemia leucocytes. Differences be-

tween the electrometric-titration curves of these preparations were also found (Polli, Di Majorca, Rosoff & Cavalieri, 1958). These results indicate that the deoxyribonucleic acid samples differ in their resistance to denaturation (see Rice & Doty, 1957). Since the base composition of the samples from all these sources was found to be the same, it would appear that the variations in the ease with which the samples can be denatured are due to differences in the physical structure of the molecules. In an attempt to determine the nature of these differences Polli & Fasoli (1956) studied the sedimentation characteristics of a variety of samples of deoxyribonucleic acid, including preparations from leucocytes of myeloid leukaemia. The results of their experiments, which were performed with concentrations in the range 0.22–0.01 %, failed to indicate any differences between the samples. It was, however, appreciated that at the lowest concentration used considerable interaction between the molecules still occurs and that this might obscure possible differences. Sedimentation experiments have now been performed at lower concentrations and the results obtained show significant differences between the samples of deoxyribonucleic acid from normal and leukaemic leucocytes.

EXPERIMENTAL

Three of the samples were prepared by the method of Gulland, Jordon & Threlfall (1947) as modified by Chargaff (1955). The remainder were prepared by the method of Kay, Simmons & Dounce (1952) with the modifications suggested by Chargaff (1955) (see Table 1). During the course of the preparation 0.01M-NaCl was used instead of water as a solvent to prevent denaturation. For the experiments to be reported solutions were obtained by dissolving the deoxyribonucleic acid (DNA) in 0.01M-NaCl and then adding an equal volume of 0.4M-NaCl. These solutions were then further diluted with 0.2M-NaCl as required. No attempt was made to maintain a constant pH in these experiments, since Alexander & Stacey (1955) have shown that the shape of DNA molecules changes very little in the range pH 4–7. The sedimentation experiments were performed in a Spinco analytical ultracentrifuge fitted with an ultraviolet-light-absorption optical system. From the results obtained at a series of concentrations in the range 0.01–0.001 % of DNA, the curves giving the distribution of sedimentation coefficients were calculated in the manner described by Shooter & Butler (1956). From these curves the curves giving the distribution of sedimentation coefficients at infinite dilution were obtained by the method of Oth & Desreux (1955).

It has been found that with calf-thymus DNA the presence of aggregates cross-linked by small amounts of residual protein may profoundly affect the shape of the distribution curve. Treating the samples with chymotrypsin considerably reduced the proportion of such aggregates (Butler, Phillips & Shooter, 1957). To determine whether the different shapes of the distribution curves observed in the present work were due to the presence of

varying amounts of protein-linked aggregates, the effect of treating some of the samples with chymotrypsin was investigated. Solutions of samples 1–10 in 0.2M-NaCl were prepared at four different concentrations, and each solution was divided into two parts. To one part of each a small amount of crystalline chymotrypsin was added. The solutions were shaken to dissolve the enzyme and were then stored at 4° for 1–3 days (these conditions were the same as those used in the experiments with calf-thymus DNA). Sedimentation experiments were performed with each of these solutions and the sedimentation-coefficient distribution curves were calculated. It was found that in all cases the distribution curves observed in the presence and in the absence of the enzyme were identical.

RESULTS

The curves giving the distribution of sedimentation coefficients at infinite dilution of the 16 samples of DNA investigated are shown in Figs. 1–5; some of the characteristics of these curves are given in Table 1. The curves in the figures are the integral distribution curves: the ordinate corresponding to a sedimentation coefficient S_0 gives the fraction of DNA with sedimentation coefficient $\leq S_0$, and the slope of the line at the point gives the weight fraction of DNA with sedimentation coefficient S_0 . It can be seen that the samples of DNA from the leucocytes of lymphatic leukaemia prepared by the method of Gulland *et al.* (1947) (Fig. 1, A) are quite different from those prepared by the method of Kay *et al.* (1952) (Fig. 1, B). The liberation of DNA from calf-thymus nucleoprotein with these two methods has been studied by Shooter & Butler (1957). It was found that when chloroform was used to denature and remove protein (Gulland *et al.* 1947) a marked fractionation of the DNA occurred, much of the material with high rate of sedimentation being lost. When, however, the protein was removed from the nucleoprotein complex with dodecyl sulphate (Kay *et al.* 1952) the distribution curve of the product was almost identical with that of the DNA in the original nucleoprotein. The method of Gulland *et al.* cannot therefore be regarded as reliable. The results obtained for the three samples of DNA from leucocytes of lymphatic leukaemia with the method of Gulland *et al.* will not therefore be considered further in the discussion.

It can be seen from the figures that, except for the samples from leucocytes of myeloid leukaemia (Fig. 2), the reproducibility of the preparations from each source is good. The curves for the different samples from normal leucocytes (Fig. 3) and the leucocytes of lymphatic leukaemia (Fig. 1) do not differ by more than ± 1.2 s over the whole range of the distributions. The two samples from non-leukaemic spleen, 4MD and 5MD (Fig. 4), show even better agreement, the curves differing by

less than ± 0.5 s. The distribution curves for preparations from myeloid leukaemia leucocytes differ by as much as ± 3 s (Fig. 2).

DISCUSSION

If the weight average sedimentation coefficients of the different samples (Table 1) are compared, it is found that for deoxyribonucleic acid from normal leucocytes $S_{av}^0 = 26.8-27.0$ s; preparations from leucocytes of lymphatic leukaemia (excluding samples 3, 4 and 6, see above) give higher values, $S_{av}^0 = 28.4-30.8$ s, and preparations from leucocytes of myeloid leukaemia, with the exception of sample 10, give lower values, $S_{av}^0 = 22.8-27.0$ s. These results indicate that it is in general possible to distinguish between normal and leukaemic leucocytes in terms of the sedimentation characteristics of the deoxyribonucleic acid. Two problems are raised: (a) what is the significance of the differences between normal and leukaemic deoxyribonucleic acid and (b) why do preparations from leucocytes of myeloid leukaemia exhibit a lack of

reproducibility whereas samples from the other sources studied are reproducible?

Histological investigations (Wintrobe, 1956*a*) have shown that in chronic lymphatic leukaemia there is a striking uniformity of white cells in the blood, small lymphocytes accounting for 90-99 % of the total cells. In chronic myeloid leukaemia, on the other hand, the leucocytes in any one sample exhibit varying degrees of maturity. Myeloblasts, myelocytes, metamyelocytes and polymorphonuclear leucocytes are usually found in this latter disease, the proportions of the different cells varying from patient to patient. The preparations of

Fig. 1. Sedimentation-coefficient distribution curves at infinite dilution for DNA from lymphatic leukaemia leucocytes. Group A: prepared by the method of Gulland *et al.* (1947); $\cdots$, 2; $---$, 4; $- \cdot -$, 6. Group B: prepared by the method of Kay *et al.* (1952); $\cdots$, 1; $- \cdot -$, 3; $---$, 5.

Fig. 2. Sedimentation-coefficient distribution curves at infinite dilution for DNA from myeloid leukaemia leucocytes: $-$, 7; $---$, 8; $\cdots$, 9; $- \cdot -$, 10. 10 was from a patient before treatment, 7 from the same patient after treatment with Myleran; 8 and 9 were from two other patients.

Fig. 3. Sedimentation-coefficient distribution curves at infinite dilution for two preparations of DNA from normal leucocytes: $---$, 20 LD; $\cdots$, 19 LD.

Fig. 4. Sedimentation-coefficient distribution curves at infinite dilution for DNA from spleen: $- \cdot -$, 3 MD (Brill-Simmons disease); $\cdots$, 4 MD (normal); $---$, 5 MD (aplastic anaemia).

Fig. 5. Sedimentation-coefficient distribution curve at infinite dilution for DNA of the leucocytes from tuberculous empyema, 21 LD.

Table 1. Comparison of the weight average sedimentation coefficients at infinite dilution and the quartile deviation of the distributions of the preparations of deoxyribonucleic acid

Sample	Source	Average sedimentation coefficient (s)	Quartile deviation (s)
19LD	Leucocytes, normal	27.0	12.1
20LD		26.8	9.6
2	Leucocytes, lymphatic leukaemia*	18.0	4.6
4		19.4	5.6
6		20.6	5.2
1	Leucocytes, lymphatic leukaemia	30.8	12.4
3		29.5	12.1
5		28.4	12.0
10	Leucocytes, myeloid leukaemia	27.0	12.4
7		24.5	11.6
8		22.8	11.0
9		22.8	8.6
4MD	Spleen, normal	26.2	7.8
5MD	Spleen, aplastic anaemia	26.8	8.2
3MD	Spleen, Brill-Simmons disease	29.8	11.6
21LD	Leucocytes from tuberculous empyema	24.6	8.4

* Prepared by the method of Gulland *et al.* (1947).

Fig. 6. Comparison of the arithmetic average distribution curves for DNA from the different sources: —, normal leucocytes; ---, non-leukaemic spleen; ···, spleen (Brill-Simmons disease); — — —, lymphatic leukaemia leucocytes; — · — · —, myeloid leukaemia leucocytes.

deoxyribonucleic acid from leucocytes of myeloid leukaemia which have been investigated were obtained from three patients. Preparations 7 and 10 were obtained from the leucocytes of one patient, 10 before treatment and 7 at a later stage after treatment with Myleran (busulphan). Myleran therapy (Wintrobe, 1956b) induces marked changes in the blood picture. The proportion of immature cells decreases and the number of polymorphonuclear leucocytes increases. The four samples of deoxyribonucleic acid were therefore obtained from cell populations differing both in composition and maturity. The observed changes in the sedimentation characteristics of the deoxyribonucleic acid preparations from leucocytes of myeloid leukaemia might be due to (a) variations associated with the average degree of maturity of the cells,

(b) variations due to the presence of different proportions of the types of cells or (c) the occurrence of specific changes of the deoxyribonucleic acid of all the cells. If the last hypothesis is correct it would require the additional postulate that Myleran modifies the physicochemical characteristics of the deoxyribonucleic acid of some of the leucocytes. On the basis of the present results it is not possible to distinguish between these three possibilities. The implications of the first two hypotheses are of some interest. If the properties of the deoxyribonucleic acid of a cell varied with the degree of maturity the changes must be induced by metabolic processes occurring within the cell. If the average characteristics of deoxyribonucleic acid preparations vary with the proportions of different white cells then the characteristics of the deoxyribonucleic acid in the different types of cells cannot be the same. Were either of these hypotheses correct problems of considerable genetic importance would be raised. The reproducibility of the deoxyribonucleic acid preparations from normal leucocytes and from leucocytes of lymphatic leukaemia would appear to be, in each case, a reflexion of the uniformity of the cell population. The cell populations of normal and leukaemic blood are not the same. The differences observed in the sedimentation characteristics of the deoxyribonucleic acid from these two sources may therefore be explained in terms of either of the hypotheses (b) and (c) above. To distinguish between these two possibilities the properties of the deoxyribonucleic acid of the different types of leucocytes will have to be studied.

If the distribution curves of sedimentation coefficients for deoxyribonucleic acid from normal leucocytes and non-leukaemic spleen in Fig. 6 are compared it will be seen that the lower halves of the curves, from 10 to 27 s, are nearly the same but that the curves differ considerably in the range 27–50 s. In view of the reproducibility observed for the two preparations from each of these sources, the results indicate that the heterogeneity of deoxyribonucleic acid in leucocytes and in spleen is not the same. It may be noted that Bendich, Pahl & Beiser (1956) have demonstrated by chromatography experiments that deoxyribonucleic acid preparations from rat brain and kidney are different. These results suggest that deoxyribonucleic acid may be tissue specific.

Of the three samples of deoxyribonucleic acid from spleen that have been investigated, two (4MD and 5MD) give almost identical distribution curves but the third (3MD, Brill-Simmers disease) is markedly different (Fig. 4). Comparison of the curves of Fig. 6 shows that the distribution observed for 3MD is very similar to that for the deoxyribonucleic acid of leucocytes of lymphatic leukaemia. Brill-Simmers disease frequently develops into lymphatic leukaemia. At the time of the splenectomy histological and clinical investigations did not show the presence of lymphatic leukaemia, but the patient has since developed this disease. The sedimentation results therefore suggest that a change in the nature of the deoxyribonucleic acid to a form which is found in the leucocytes of lymphatic leukaemia has occurred within the spleen at a stage before that at which the disease can be diagnosed by the normal clinical and histological techniques. The implications of this in relation to the development of the disease are being further studied.

It would be of considerable value to determine whether the differences observed between the distribution curves of the various samples studied are due to variations of the molecular weight or the shape of the molecules. It has been shown that the degradation of deoxyribonucleic acid by deoxyribonuclease results in a decrease in molecular weight of the particles without any significant change in shape (Thomas, 1956). Sedimentation experiments (Shooter, 1958) have shown that the action of this enzyme on deoxyribonucleic acid results in a progressive movement of the distribution curve to lower sedimentation coefficients, but the shape of the curve (i.e. the ratio of the weight average sedimentation coefficient to the quartile deviation) is not altered. The shapes of the distribution curves of the samples investigated do not vary very greatly, suggesting that the samples differ in respect of the molecular weight of the particles rather than in their shape. With the deoxyribo-

nucleic acid from the leucocytes of tuberculous empyema (Figs. 5, 6) it is known that the leucocytes undergo nucleolysis and other degradative processes within the pleural cavity.

Investigations of preparations of deoxyribonucleic acid from a variety of sources (Butler & Shooter, 1958) has shown that the quartile deviations of the distributions of sedimentation constants for mammalian deoxyribonucleic acid (calf-thymus, rat-liver) are in the range 12–13 s units, whereas simpler organisms (*Haemophilus influenzae*, T2 and D4 bacteriophage) have quartile deviations in the range 6–10 s units. Of the present series of samples (Table 1), nine have quartile deviations in the range 10–13 s units. Of the exceptions, 2, 4 and 6 may be disregarded since they have been subjected to considerable fractionation during preparation, 21LD is a degraded form of a normal deoxyribonucleic acid and 9 may also be a degraded deoxyribonucleic acid. The two spleen samples (4MD and 5MD) appear to be quite different from other preparations from mammalian sources. This difference is presumably correlated in some way with the function of the spleen. The anomaly of the spleen samples is being further investigated.

SUMMARY

1. Sedimentation-coefficient distribution curves at infinite dilution have been determined for samples of deoxyribonucleic acid from human leucocytes and spleen.
2. The distributions for preparations from normal leucocytes and non-leukaemic spleen indicate that deoxyribonucleic acid exhibits tissue specificity.
3. Differences exist between the distributions observed for deoxyribonucleic acid from normal leucocytes and from leucocytes of myeloid and lymphatic leukaemia.
4. In Brill-Simmers disease changes in the deoxyribonucleic acid of the spleen to a form which is found in the leucocytes in lymphatic leukaemia can occur before the stage at which lymphatic leukaemia can be diagnosed clinically.
5. The deoxyribonucleic acid of leucocytes from tuberculous empyema appears to be a randomly degraded form of that occurring in normal leucocytes.

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The Separation of Flavins by Paper Electrophoresis and its Application to the Examination of the Flavin Contents of Micro-organisms

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Extracts of *Clostridium kluyveri* are a deep green-brown, but under hydrogen this colour disappears leaving a faint pink colour (Peel & Barker, 1956). Substantially the same observations hold for the rumen organism LC1 of Elsden, Volcani, Gilchrist & Lewis (1956). It appeared likely that the pigments concerned were flavin derivatives, and the presence of such apparently large amounts in these two strict anaerobes was of particular interest. Since very little data were available on the occurrence of flavins in micro-organisms a survey of a variety of species seemed a worthwhile project.

At the outset, suitable analytical methods were not available. It was considered essential that the principal flavin derivatives should be separated from each other in sufficient quantities for confirmatory tests to be applied and in such a manner as to permit an estimate of the amount of each flavin present in the organisms examined. A separation on the required scale has been accomplished by paper electrophoresis and this separation has been combined with a preliminary extraction and concentration procedure adapted from the methods used by Dimant, Sanadi & Huennekens (1952) in the preparation of flavinadenine dinucleotide. This paper describes the method and the results obtained, preliminary accounts of which have already been given (Peel, 1954, 1955).

The electrophoretic separation of flavins has since been reported from a number of laboratories

(Siliprandi, Siliprandi & Lis, 1954; Cerletti & Siliprandi, 1955; Gaudiano & Cingolani, 1954; Yagi & Matsuoka, 1955; Walaas & Walaas, 1956).

MATERIALS

Flavin derivatives. Riboflavin was obtained from L. Light and Co. (Colnbrook, Bucks); flavin mononucleotide (FMN) was a gift from Sigma Chemical Co.; riboflavinyl glucoside and one sample of flavinadenine dinucleotide (FAD) were gifts from Dr L. G. Whitby. A crude mixture of FMN and FAD was obtained from baker's yeast by following the procedure of Dimant *et al.* (1952) to the stage before partition chromatography. These products were used without further treatment as markers for paper chromatograms. For recovery experiments riboflavin, FMN and FAD were purified by paper electrophoresis as described later. Lumiflavin was prepared from riboflavin as described by Warburg & Christian (1938). Lumichrome (Karrer, Salomon, Schöpp, Schlittler & Fritzsche, 1934) was prepared as follows: a saturated solution of riboflavin in 0.01 M-ammonium formate-formic acid buffer, pH 4.6, was kept for about 7 days in a shallow layer in daylight at room temperature; HCl was then added to a final concentration of about 0.5 M and the mixture extracted with a half volume of CHCl_3 . The extract was evaporated to dryness and the residue dissolved in water for use.

Micro-organisms. Sources of micro-organisms and details of the growth conditions and media employed in obtaining cells for the examination of flavin contents are given in Table 7. In experiments on the extraction procedure the large quantities of the rumen organism LC1 required were obtained as described by Walker (1958).