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Tissue Nucleic Acids

2. THE ISOLATION AND PROPERTIES OF LIVER RIBONUCLEIC ACID

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Both ribonucleic acid and desoxyribonucleic acid have recently been shown to be present in the tissues of the sheep (Davidson & Waymouth, 1944*a*), and the current view is that the ribonucleic acid is in general a cytoplasmic constituent (Mirsky, 1943; Pollister & Mirsky, 1944), while desoxyribonucleic acid is confined to the nucleus.

So far, although the presence of pentose nucleic acids has been demonstrated in many biological materials, only three such acids have been isolated and characterized. The first to be isolated was yeast ribonucleic acid, whose structure has been reviewed by Gulland (1938, 1944). In addition to *d*-ribofuranose it contains small amounts of *l*-lyxose (Gulland, Barker & Jordan, 1943; Gulland & Barker, 1943) and is usually considered to be composed of tetranucleotide units in which the bases are adenine, guanine, uracil and cytosine in equimolecular proportions. The pancreas pentose nucleic acid was considered by Jorpes (1928, 1934) to be probably a pentanucleotide containing three molecules of purine (guanine:adenine ratio = 2:1) and two of pyrimidine. Jorpes (1924) isolated the crystalline brucine salts of cytidine·and uridine phosphoric acids from hydrolysates of this nucleic acid, and concluded that the pyrimidines were cytosine and uracil.

The third pentose nucleic acid to be characterized was isolated from tobacco mosaic virus (Loring, 1939; Cohen & Stanley, 1942). It appears to be very similar to yeast ribonucleic acid and on hydrolysis has yielded guanine, adenine, cytosine and the brucine salt of an acid similar to, and probably isomeric with, yeast uridylic acid (Loring, 1939).

Liver tissue contains a desoxyribonucleic acid very similar to thymus desoxyribonucleic acid (Levene, 1922; Peters, 1911; Ishiyama, 1928; Greenstein & Jenrette, 1940). This nucleic acid has been prepared from isolated liver nuclei by Dounce (1943). The cytoplasm of liver cells contains particles of at least two sizes (Claude, 1941, 1943*a*, *b*; Bensley, 1942) composed of phospholipin nucleoprotein complexes containing a pentose nucleic acid which has not yet been isolated and characterized.

It has previously been shown that sheep liver is a tissue with a relatively high content of pentose nucleic acid (Davidson & Waymouth, 1944*a*). From a dry ethanol-ether powder of sheep liver the nucleic acids were extracted with 10 % sodium chloride and precipitated as lanthanum salts. The precipitate was rich in pentose. Moreover, the sodium chloride extract contained a substrate for the enzyme ribonuclease, and the amount of this substrate, in terms of phosphorus, indicated a ribonucleic-acid content of the same order as that found by the pentose estimations. The evidence for the presence of a pentose nucleic acid, presumably a ribonucleic acid, in liver is thus fairly conclusive (Davidson & Waymouth, 1944*b*), but it has seemed desirable that this nucleic acid should be isolated and characterized.

METHODS

Principle. For the extraction of the nucleic acids mild methods are essential and the use of alkali has been avoided. The methods usually employed for the preparation of desoxyribonucleic acid are sufficiently drastic to break down much of the ribonucleic acid. Extraction with 10% NaCl as used by Clarke & Schryver (1917) for the preparation of yeast ribonucleic acid has proved satisfactory provided that the liver proteins were first denatured with ethanol. Extraction of fresh minced liver with 10% NaCl gave tissue extracts from which separation of the nucleic acids was difficult.

From the 10% NaCl extract, the nucleic acids were precipitated with ethanol. The crude pentose nucleic acid was purified through the barium salt by the method of Jorpes (1934) and was finally precipitated from glacial acetic acid.

Procedure. Fresh sheep liver was minced, mixed well with 3 vol. of ethanol, and allowed to stand overnight. The ethanol was sucked off on a large Buchner funnel and the tissue suspended in two further successive portions of ethanol. After being washed with ether, it was dried in air and ground to a fine powder in a mill. 200 g. portions of this powder were suspended in 800 ml. 10% NaCl and allowed to stand overnight in the refrigerator. The suspension was then filtered through muslin and the solid residue mixed with 400 ml. 10% NaCl, heated for 30 min. in a boiling water-bath, and cooled. The mixture was again filtered through muslin and the residue washed with a further 200 ml. 10% NaCl at room temperature and filtered. The filtrates were combined (1000 ml.) and centrifuged. A small amount of precipitate and of floating fatty material was discarded. To the slightly cloudy fluid 2 vol. of ethanol were slowly added with brisk stirring, and the mixture allowed to stand overnight in the refrigerator. As much as possible of the supernatant fluid was then siphoned off and the precipitate centrifuged down, washed once with 70% ethanol, twice with absolute ethanol, dried in a vacuum desiccator, and powdered. Yield 4–5 g.

4 g. of this crude nucleic acid were ground in a mortar with 60 ml. water and centrifuged. The residue was ground with 40 ml. water and centrifuged. To the combined supernatant fluids, 0·25 vol. 20% barium acetate (pH 6·8) was added and the precipitate of barium salts centrifuged down and washed three times with 5% barium acetate. The precipitate was then ground in an ice-cold mortar with 4–5 ml. ice-cold N-HCl and centrifuged. The precipitate was washed with successive 3 ml. portions of ice water until the washings were free from Ba^{++} ions, and ice-cold N-NaOH added dropwise until the solution was no longer acid. Alkaline reaction was carefully avoided. The solution was made up to about 10 ml. with ice-water and made just acid with acetic acid. A small precipitate was centrifuged down and discarded. The supernatant fluid was poured into 10 vol. glacial acetic acid with constant stirring. The precipitate was centrifuged down, washed three times with ethanol, once with ether and dried *in vacuo*. Yield 200–250 mg.

500 mg. of this material were cautiously dissolved in water with the addition of the minimum amount of ice-cold alkali. A small amount of material remained undissolved. The solution (10 ml.) was made just acid with acetic acid and centrifuged. The supernatant fluid was poured into 10 vol. glacial acetic acid, washed twice with ethanol, once with ether, and dried *in vacuo*. Yield 300 mg.

Properties of the nucleic acid

The liver ribonucleic acid prepared in this way is a light brown powder, slightly soluble in water and giving an acid solution; it is also easily soluble in dilute alkali. It gave a negative biuret test and negative tests for desoxypentoses. Tests for pentoses were strongly positive. After drying at 100° it gave N = 15·0–15·1, P = 6·7–7·6%. Both these values are lower than the theoretical values which, for a tetranucleotide of the yeast ribonucleic acid type, $C_{38}H_{47}O_{28}N_{15}P_4$, are N = 16·3, P = 9·5%. It has been pointed out that the figures for P in many samples of nucleic acid tend to be low (Fletcher, Gulland, Jordan & Dibben, 1944; Fletcher, Gulland & Jordan, 1944). A sample of yeast ribonucleic acid precipitated twice from glacial acetic acid gave N = 15·0, P = 7·7%.

The purine content was determined by hydrolyzing a sample of the nucleic acid in 1·2N-H_2SO_4 at 100° for 1 hr. The purines were precipitated with copper hydroxide and copper bisulphite as described by Kerr & Blish (1932) and Kerr (1940), and the purine N determined. It varied in different samples from 55·1 to 61·3% of the total N. For a tetranucleotide of the yeast nucleic acid type the figure would be 66·7%, for a pentanucleotide with three purine nucleotides 75%, and for a hexanucleotide with four purine nucleotides 80%.

The easily hydrolyzable P, i.e. that derived from the purine nucleotides, was determined by hydrolyzing a portion of the nucleic acid for 2½ hr. with 5% (v/v) H_2SO_4 at 100°. Inorganic P was then determined by the method of Allen (1940). It amounted to 54·2–54·7% of the total P. For a tetranucleotide of the yeast ribonucleic acid type the figure is 53% (Jorpes, 1934), for a pentanucleotide 63%, for a hexanucleotide 69%.

The pentose content of the nucleic acid was estimated by the method of Mejbaum (1939), but with 30 min. heating as recommended by Schlenk (1942). The greenish blue colour was examined in the Hilger Spekker absorptiometer and the readings plotted against phosphorus content. The results obtained with two different samples of the liver ribonucleic acid are shown in Fig. 1. The points obtained for the two samples, and the points for yeast ribonucleic acid, lie on the same line. Liver ribonucleic acid and yeast ribonucleic acid therefore contain the same amounts of pentose relative to phosphorus.

The action of crystalline ribonuclease (Kunitz, 1940) on liver ribonucleic acid was also examined. To each of three conical 15 ml. centrifuge tubes containing 1·5 ml. of a 0·1% solution of the sodium salt of liver ribonucleic acid, 2·4 ml. veronal acetate buffer pH 6·12 were added. To one tube 0·01 mg. crystalline ribonuclease was added. To one of the remaining two (control) tubes 4·0 ml. 0·25% uranyl

acetate in 2·5% trichloroacetic acid were added immediately. The other control tube and the tube containing enzyme were incubated at 37° for 1 hr. before receiving the uranyl acetate reagent. The precipitate produced by the reagent was centrifuged down, washed with 2 ml. 0·125% uranyl acetate

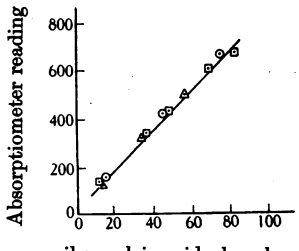

Fig. 1. Relationship between the amounts of pentose (absorptiometer reading—colorimetric method of Mejbaum, 1939) and of phosphorus found in different amounts of specimens of ribonucleic acids. △—△ Liver ribonucleic acid. ⊙—⊙ Liver ribonucleic acid. ⊡—⊡ Yeast ribonucleic acid.

in 1·25% trichloroacetic acid, dissolved in 0·5M-Na_2CO_3, and transferred to a digestion flask for total P estimation. The P content of the precipitate from the tube containing the enzyme was 39% of that of the precipitate from the control tubes, i.e. 61% of the total P has been rendered non-precipitable by the uranyl acetate reagent. A similar figure was obtained with yeast ribonucleic acid.

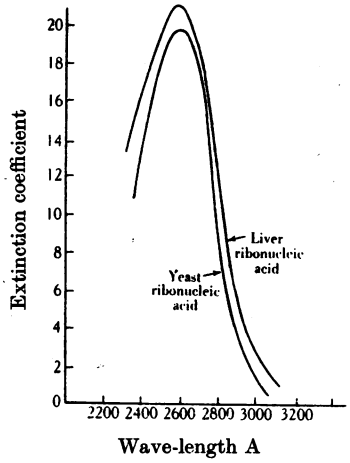

Fig. 2. Absorption spectrum of liver ribonucleic acid and yeast ribonucleic acid as sodium salts in 0·005M-phosphate buffer pH 7·2. Concentration = 0·0023% ribonucleic acid.

Absorption spectrum. The absorption spectrum of the nucleic acid was measured in a Hilger medium quartz spectrograph. We are indebted to Dr A. Clow for carrying out these measurements.

The nucleic acid was dissolved in the minimum amount of dilute NaOH and diluted to a suitable concentration (40–160 μg. P/100 ml.) with 0·005M-phosphate buffer pH 7·2. A sample of yeast ribonucleic acid (British Drug Houses Ltd.) was examined at the same time under the same conditions. The curves are shown in Fig. 2. The curve for yeast ribonucleic acid shows the typical absorption maximum in the region of 2600 A, and is similar to the curves for pentose nucleic acids shown in the papers of Caspersson (1936) (yeast and pancreas pentose nucleic acids), Lavin, Thompson & Dubos (1938) (pneumococcal pentose nucleic acid) and Lavin, Loring & Stanley (1939) (virus pentose nucleic acid). The liver ribonucleic acid gives a curve very similar to that from yeast ribonucleic acid, the maximum being even slightly higher than with the latter.

Products of hydrolysis. The products of hydrolysis of the nucleic acid were investigated, the procedure of Bredereck & Richter (1938) as used by Gulland & Barker (1943) being followed. The hydrolysis followed the same course when the process was carried out with 1 g. yeast ribonucleic acid, and 1 g. of liver nucleic acid.

Liver ribonucleic acid (1 g.) was heated with 10 ml. 2% (w/v) H_2SO_4 for 2 hr. in a flask attached to a reflux condenser and immersed in an oil-bath at 105–110°. The mixture was transferred to a centrifuge tube and the concentration of H_2SO_4 increased to 3·8% (w/v) by the addition of conc. H_2SO_4. The tube was allowed to stand overnight at room temperature. The precipitate of guanine sulphate was centrifuged down and washed with 1 ml. of 2% H_2SO_4.

To the supernatant fluid and washings $Ba(OH)_2$ solution was added until the reaction was just neutral. The $BaSO_4$ was centrifuged down and washed with a few drops of water. The supernatant and washings were taken down to 5 ml. in a shallow dish in a vacuum desiccator over conc. H_2SO_4. Solid matter which separated out was centrifuged down, washed with a few drops of water and discarded, and the supernatant fluid and washings were taken down to dryness in the vacuum desiccator. The dry residue was rubbed up with 0·5 ml. water and transferred to a centrifuge tube. Insoluble matter was centrifuged down, washed with 2 drops of water and discarded. To the supernatant and washings in a fresh centrifuge tube cooled in ice, 7 ml. pyridine were added with stirring. The cytidylic acid fraction was centrifuged down and washed twice with 1 ml. pyridine.

The supernatant and washings were taken to dryness in a vacuum desiccator over $CaCl_2$. The residue was dissolved in 2 ml. water and ethanol added until no further precipitate appeared (about 5 ml.). The precipitate was centrifuged down. The supernatant fluid was taken to dryness in a vacuum

desiccator over $CaCl_2$ and dissolved in 2·5 ml. water. A small insoluble residue was discarded. 0·5 ml. of a 2% ethanolic brucine solution was added and the tube set aside for 5 days at room temperature. The brucine salts of the uridylic acid fraction were centrifuged down.

The supernatant fluid was made alkaline with hot saturated $Ba(OH)_2$. The precipitate was discarded and the supernatant was extracted three times with chloroform to remove brucine. The aqueous layer was then centrifuged to remove a small amount of insoluble material and was exactly neutralized with 10% H_2SO_4. The $BaSO_4$ was centrifuged down and washed with a few drops of water. In the combined supernatant fluid and washings pentose was determined by the method of Mejbaum (1939). The amount present was 29·1 mg., calculated as ribose.

To 15 mg. of the pentose, dried from the frozen state in a centrifuge tube, 20 mg. *p*-bromophenylhydrazine were added, followed by 0·48 ml. water and 0·1 ml. 50% acetic acid. The mixture was allowed to stand overnight at room temperature. The yellow precipitate of the *p*-bromophenylhydrazone was centrifuged down, washed with a little absolute ethanol and recrystallized from 50% ethanol. It softened at 168° and melted at 169°. A mixture of this material with ribose *p*-bromophenylhydrazone made from pure *d*(−)-ribose (prepared from guanosine by the method of Levene & Clark (1921)) softened at 168° and melted at 169–170°. The *p*-bromophenylhydrazone made from this sample of ribose softened at 169° and melted at 171–172°. The *p*-bromophenylhydrazone made from the pentose of yeast ribonucleic acid melted at 166°. Arabinose *p*-bromophenylhydrazone prepared in exactly the same way melted at 154–155°.

The guanine sulphate precipitate was stirred with hot 10% (w/v) H_2SO_4 until no more would dissolve, and was centrifuged while hot. A moderately large insoluble residue was discarded. To the hot solution excess ammonium hydroxide solution was added. The precipitate of free guanine was centrifuged down and washed with a little dilute ammonia.

The supernatant and washings were boiled to remove ammonia, cooled and treated with saturated picric acid solution. On standing in the refrigerator overnight, a precipitate of adenine picrate appeared. It was centrifuged down and recrystallized from 25% (v/v) acetic acid.

The amounts of purine and pyrimidine bases obtained have not been sufficient for the complete characterization of all the bases.

DISCUSSION

The composition of the material under examination, the pentose content, the absence of positive tests for proteins and desoxypentose, indicate that it is a nucleic acid of the pentose nucleic acid type. Since the pentose appears to be ribose the acid can be correctly designated 'liver ribonucleic acid'.

The presence of ribose in liver tissue was suggested by Levene & Jacobs (1909), who maintained that guanylic acid from liver contained *d*-ribose: Winter (1927) identified ribose derivatives in a mixture of goat liver and muscle.

It has been generally assumed that the sugar in pentose nucleic acids and nucleotides is *d*-ribose but the evidence for this has until recently not been altogether satisfactory. Gulland & Barker (1943), using the benziminazole method (cf. Moore & Link, 1940; Dimler & Link, 1943), have now demonstrated conclusively that the sugar in yeast nucleic acid and its related nucleotides is *d*-ribose. This method of pentose identification is probably superior to any other, but it requires a larger amount of sugar than has been available to us in the present investigation. The *p*-bromophenylhydrazone method requires much less pentose and has been used by Schlenk (1942) to show that the sugar in cozymase is *d*-ribose. The melting-points for ribose-*p*-bromophenylhydrazone quoted in the literature (cf. Levene & Jacobs, 1909) vary widely. In the present instance, however, the agreement between the melting-points of the *p*-bromophenylhydrazones prepared at the same time under the same conditions from ribose and the pentose of the liver nucleic acid and recrystallized from the same solvent, together with the results of the mixed melting-point, is sufficient to justify the conclusion that the pentose is ribose. The melting-point of the *p*-bromophenylhydrazone of arabinose is lower than that of ribose, and that of the xylose derivative is lower still. The possibility of the presence of small amounts of *l*-lyxose in the liver ribonucleic acid such as are present in yeast ribonucleic acid (Gulland & Barker, 1943) cannot be excluded, but the amounts of nucleic acid available have been too small for any attempt to be made to detect it.

The similarity between liver ribonucleic acid and yeast ribonucleic acid is revealed by the pentose content, by the absorption spectrum, by the action of ribonuclease, and by the nature of the products of hydrolysis. Moreover, the purine content and the easily hydrolyzable P content suggest that the liver ribonucleic acid, like yeast ribonucleic acid, is composed of tetranucleotide units containing equimolecular amounts of purines and pyrimidines. It is possible, therefore, that liver ribonucleic acid may differ from the pentose polynucleotide of the pancreas, which may be a pentanucleotide containing three purine nucleotide residues (Jorpes, 1934).

SUMMARY

1. A method for the isolation of a ribonucleic acid from liver tissue is described.

2. The nucleic acid is free from protein and from desoxypentose. It resembles yeast ribonucleic acid in its pentose content, in its absorption spectrum, in its action as a substrate for ribonuclease and in its content of purine and easily hydrolyzable phosphorus. Among the hydrolysis products *d*-ribose has been identified as the *p*-bromophenylhydrazone.

We should like to thank Dr Neil Campbell of Edinburgh University for supplying us with a specimen of pure *p*-bromophenylhydrazine, and for his advice on the preparation of hydrazones. This work was carried out during the tenure of a Carnegie Teaching Fellowship by one of us (J.N.D.). A grant for scientific assistance from the Medical Research Council and an expenses grant from the Carnegie Trust for the Universities of Scotland to one of us (J.N.D.) are gratefully acknowledged.

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Tissue Nucleic Acids

3. THE NUCLEIC ACID AND NUCLEOTIDE CONTENT OF LIVER TISSUE

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It has been shown previously (Davidson & Waymouth, 1944 *a*, *d*) that ribonucleic acids are widespread in animal tissues and are present in high concentrations in rapidly growing embryonic tissues, although the proportion of ribonucleic acid to desoxyribonucleic acid appears in most cases to be similar in the embryo and the corresponding adult tissue. A ribonucleic acid has been isolated from the liver of the sheep and its properties described (Davidson & Waymouth, 1944*b*). It has been demonstrated histochemically in the cytoplasm of the liver cell (Davidson & Waymouth, 1944*c*). It is of some interest, therefore, to determine the amount of this nucleic acid present in liver tissue and its possible variation under different circumstances. For such an examination liver tissue has many advantages. It is possible to compare normal adult liver not only with the embryonic organ but with the regenerating organ and with liver tumours produced by the administration of such agents as dimethylaminoazobenzene (butter yellow). This fact has already been utilized by Burk (1942) in an