Preliminary Notes

A lipid factor from liver which enables *P. notatum* extracts to break down intact lecithin

FAIRBAIRN[1] reported that a dialysed extract of *Penicillium notatum* possessed an active phospholipase B which attacked lysolecithin but was completely inactive towards intact lecithin. In the present experiments it was found that when similar phospholipase B preparations were incubated with crude liver-lipid emulsions at pH 4, the lecithin contained in them was rapidly and completely degraded although purified ovo-lecithin or liver lecithin were not attacked by the enzyme. Analysis of the reaction mixture for glycerylphosphorylcholine, acid-soluble P, fatty acids and disappearance of fatty-acid acyl ester bonds indicated that the lecithin had been broken down into glycerylphosphorylcholine and fatty acids. Paper chromatography confirmed the formation of glycerylphosphorylcholine, and also showed that phosphatidylethanolamine had been attacked with the liberation of glycerylphosphorylethanolamine.

By chromatography on alumina columns and solvent fractionation, etc., a purified fraction has been isolated from sheep and rat liver, which is especially active in causing extracts of *P. notatum* to attack purified lecithin. No lecithin attack was detected either in the absence of the fraction or the enzyme preparation. The fraction is free of lecithin, but contains some phosphatidylethanolamine and a little phosphatidyl inositol. In solubility it behaves completely as a lipid, being soluble in diethyl ether, chloroform and absolute methanol, and is not removed from $CHCl_3$ by aqueous washing according to the procedure of FOLCH *et al.*[2]. The active fraction is thermostable at 100° (pH 4), and also when left standing in methanolic solution at room temperature, and it is insoluble in acetone. It is rapidly destroyed by boiling with 0.1 *N* acids or alkalis or by precipitation of the tissue with trichloroacetic acid.

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On a chemical degradation of deoxyribosenucleic acid

Although degraded to mononucleotides by a combination of enzymes[1] DNA has been well known for its resistance to chemical hydrolysis. We wish to report here a method by which DNA has been degraded with alkali under mild conditions.

The method employed was suggested by the hydrolytic behavior of ribosenucleic acid, RNA, which in contrast to DNA is readily hydrolyzed to mononucleotides by alkali. This surprising difference in hydrolytic behavior has been very elegantly explained by BROWN AND TODD[2] on the basis that RNA can exist as a cyclic 2′, 3′-ribose phosphate, a tertiary ester sensitive to hydrolysis, whereas DNA, lacking the 2′-hydroxyl, can exist only as a secondary phosphate ester relatively stable to alkali. Esterification of DNA to the sensitive tertiary phosphate state should presumably, therefore, give a product hydrolyzable by alkali.

The procedure followed was based upon a previous report[3] that naphthyl acid phosphates are quantitatively esterified by diazomethane. The sodium "half-salt" of β-naphthyl dihydrogen phosphate, for example, gave with diazomethane an equimolar mixture of β-naphthyl dimethyl phosphate and sodium β-naphthyl methyl phosphate in essentially quantitative yield. Subsequently, phosphatoethyl cotton[4]*, an ion-exchange fabric, was similarly quantitatively methylated by this procedure[5]. More recently BROWN AND TODD[6] prepared alkyl esters of the four ribose nucleotides as well-characterized products by the use of diazoalkanes, and the dimethyl ester of uridine-3-phosphate with diazomethane[7].

* A sample of this material had very kindly been made available in an other connection by Dr. JOHN D. GUTHRIE of the Southern Regional Research Laboratory.

Comparable methylation of DNA in the acid form, would transform the alkali-stable dinucleoside phosphates in the molecule to alkali-labile dinucleoside methylphosphate groups. Subsequent cleavage, on treatment with alkali, at the internucleotide links (bond a or b, Fig. 1), would degrade the molecule to mononucleotides. In partially neutralized DNA's, moreover, phosphoric acid groups in the salt form would be protected, and methylation, and hence hydrolysis, would take place exclusively at phosphate groups in the acid form, as indicated (Fig. 1).

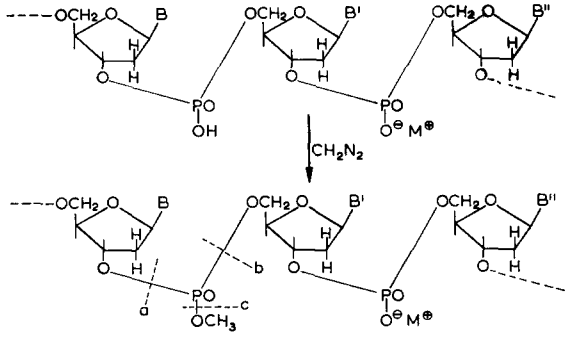

Fig. 1.

Hydrolysis at the internucleotide bonds in the latter cases would give oligonucleotides of various chain lengths.

Thus if hydrolysis takes place in the manner indicated, as the results below suggest, polydeoxynucleotide chains can be cleaved in a predetermined way to give fragments of predictable average chain length. In this regard this method is of special significance in studies concerned with the molecular structure of DNA.

In this initial study crude commercial DNA, precipitated from solution in 1 *M* saline with a slight excess of hydrochloric acid, was used. From the potentiometric titration curve of the acidified product it was evident that 30% of the ionizable primary phosphoric acid groups were still in the salt form (neutral equivalent, calculated: 340; found: 500) and that 13% of the secondary phosphoric acid groups were ionizable. The latter observation indicates that this sample consisted of polynucleotide chains, eight nucleotide units in length on the average.

This acidified DNA in finely divided form, treated twice with a total of an 8 molar excess of diazomethane in ether, gave a neutral product (pH 6.6) in which the acidic primary and secondary phosphoryl groups were apparently quantitatively methylated (calculated for DNA (0.7 equivalents primary + 0.13 equivalents secondary phosphate): methyl ester: OCH_3, 7.30%; found: OCH_3, 7.15%). When a solution of this methylated DNA in a known volume of 2.0 *N* sodium hydroxide was heated to 35° for 24 hours, and the added alkali exactly neutralized with hydrochloric acid, the resultant mixture was acidic, pH 3.0, and smelled strongly of methanol. From potentiometric titration it was apparent that treatment with alkali not only had liberated approximately the theoretical 0.7 equivalents of primary phosphoric acid as expected, but, in addition, and most significantly, approximately 0.75 equivalents of secondary phosphoric acid.

The production of 0.7 equivalents of primary phosphoric acid does not in itself imply scission of the polynucleotide chain since hydrolysis of the methyl esters (bond C, Fig. 1), might have occurred with regeneration of the original DNA. The liberation of the 0.75 equivalents of secondary phosphoric acid, however, can only mean that the chain was broken at a number of points (bond a or b, Fig. 1). The simplest interpretation of these facts is that cleavage of the methylated intranucleotide phosphate links at either bond a or b (Fig. 1) had occurred first, and essentially quantitatively; and that this cleavage was followed by a second, essentially quantitative, hydrolysis of the methyl nucleotides thus formed at either of the remaining bonds.

The hydrolytic products, consequently, should consist of a mixture of oligonucleotides and either mononucleotides and methanol or mononucleosides and methyl phosphoric acid. On the basis of random cleavage of 75% of the intranucleotide links in a "statistical" octanucleotide chain, the calculated composition of the hydrolytic mixture would be mononucleotides, 59%; dinucleotides, 33%; and trinucleotides, 8%; with an average chain length of 1.5 nucleotide units for the mixture. The average chain length for the hydrolytic products, calculated from the titration data, 1.33 nucleotide units, is, therefore, in reasonably good agreement with this predicted value.

The behavior of the hydrolysate on dialysis was also consistent with the predicted composition for the mixture. From potentiometric titration data the 35% of the mixture that was not dialyzable consisted of di- to tri-nucleotide fragments on the average (predicted size, 2.2 nucleotide units). The remaining 65% which did pass through the membrane was by implication principally mononucleotide fragments in approximately the predicted amount.

The original non-methylated DNA, in contrast, when treated identically was not appreciably hydrolyzed; 0.23 equivalents of secondary phosphoric acid groups were liberated, and 90% of the hydrolytic products were found to be undialyzable. The amount of dialyzable material, 10%, was again generally in accord with the calculated mononucleotide content of the hydrolytic products.

It is evident, therefore, from these results, that when methylated as described DNA becomes much more sensitive to alkaline hydrolysis than is normally the case. The data imply that methylation and hydrolysis are essentially quantitative but confirmation of the suggested mechanism or elucidation of the exact course of these transformations along other lines will have to await chemical identification of the hydrolytic products, on which work is currently in progress.

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Incorporation of radioactive uridine-5'-monophosphate into ribonucleic acid by soluble mammalian enzymes

Recent work from this laboratory has indicated the requirements for the degradation of uracil by enzymes of rat liver[1]. In addition, the existence in this tissue of enzyme systems which can convert uracil to uridine, uridine-5'-mono-, di-, and tri-phosphates (UMP, UDP and UTP respectively)[2] has been demonstrated. Uracil, uridine and UMP have been shown, under certain conditions, to be incorporated into the ribonucleic acid (RNA) of rat liver slices at rates comparable to those obtained with orotic acid[3]. A logical extrapolation of this work was to attempt to obtain a cell-free, and if possible, a soluble system from rat liver capable of incorporating UMP into RNA. Recently this has been achieved. The object of this communication is to describe the experimental conditions and the requirements of the system.

Dialyzed acetone powder extracts of the particle-free cytoplasmic fraction of rat liver[1] have been used throughout these experiments. The UMP-4-^{14}C was prepared from orotic acid-4-^{14}C (obtained from Tracerlab Inc.) by the method already described for the preparation of UMP-2-^{14}C[3]. At the end of the incubation period, perchloric acid was added to the incubation mixture; the precipitate obtained was washed[4] and incubated overnight in 0.2 *N* KOH at 38°; the resultant solution was acidified to 0.1 *N* with respect to perchloric acid. After centrifugation, the supernatant fraction was heated at 100° for one hour to hydrolyze the purine nucleotides, and the uridylic acid was isolated by paper chromatography[5]. Because of the low amounts of RNA present in the acetone powder extract, carrier RNA was added to the acid-denatured incubation mixture, when the extract was incubated in the absence of microsomes. This was done in order to facilitate the subsequent isolation of the uridylic acid from RNA. It was found most convenient to add the carrier RNA in the form of a known volume of rat liver microsomes to the already denatured incubation mixture. The uridylic acid was then isolated as previously described. A correction for the added carrier RNA was applied to the calculation of the specific activity of the uridylic acid isolated from RNA.

As can be seen from Table I, the microsomes alone showed no incorporation of UMP. Some activity was obtained when the microsomes were incubated in the presence of the acetone powder extract, while the latter alone showed maximal activity. Pre-incubation of microsomes alone for 10 minutes, followed by a further 10 minute incubation with the extract showed no incorporation of UMP into RNA; this value should be compared to that obtained when the extract was incubated with microsomes and ATP. This suggests that a factor is released from the microsomes which inhibits the incorporation of UMP into RNA. High-energy phosphate is essential; although ATP showed maximal activity in this system, ADP was also active.

A time curve of UMP-incorporation (not presented) into RNA in the presence and absence of microsomes shows that in the former system the specific activity attained a maximum at 10 minutes, which was then followed by a sharp decline. In contrast, in the latter system, the specific activity attained a maximum at 20 minutes; this was followed by a slower decline. It may be suggested that the inhibition exerted by the microsomes, and the decrease in the specific