STUDIES IN THE DETERMINATION OF NUCLEOTIDE SEQUENCE IN DEOXYPENTOSENUCLEIC ACIDS

by

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There have been several reports of investigations of the nucleotide sequence in pentosenucleic acids (PNA)[1] and deoxypentosenucleic acids (DNA)[2] using enzymic methods of degradation. As yet, few definite conclusions can be drawn from these experiments and only limited information has been obtained about the sequence of the pyrimidine nucleotides. Moreover, the interpretation of the results has been rendered uncertain by the possible occurrence of exchange reactions[3]. It was considered desirable, therefore, to study the possibility of using specific chemical methods of degradation in order to obtain information about nucleotide sequence in DNA.

From the work of LI, OVEREND AND STACEY[4], where there was an indication that in DNA degraded with dilute acid some deoxyribose residues were in the aldehydo form, it appeared that it would be possible to treat DNA with thioglycollic acid and thus replace the purines by carboxymethylmercaptal groups (Fig. 1). The DNA thus treated would have the deoxyribose moieties, which were originally bound to the purines, in the open-chain form and hence a hydroxyl group would be adjacent to the 3′ and 5′ phosphates. According to the theories of TODD[6] such a structure would be labile to alkali and hence, by alkaline degradation, fragments containing the pyrimidine nucleotide portions intact should be obtained. From Fig. 1 and by analogy with the case of PNA, it can be seen that fission could apparently take place in two ways represented by *a* and *b*. In either case, pyrimidine polynucleotides would be produced containing a terminal 2-deoxy-D-ribosephosphate di(carboxymethylmercaptal) group. If fission occurred at both *a* and *b*, then a sulphur-free pyrimidine polynucleotide would be obtained.

Thioglycollic acid (98%) was used for this work because it readily dissolved DNA, thus ensuring a homogeneous reaction, and was a very powerful mercaptalating agent which did not need the addition of strong acid as a catalyst. Thioglycollic acid alone, at 37° C, introduced only 50% of the theoretical sulphur into thymus DNA, but by using zinc chloride and sodium sulphate as catalysts the purines were almost completely replaced by mercaptal groups. After treatment of thymus DNA (1 g) with thioglycollic acid (45 ml), fused zinc chloride (5.5 g) and anhydrous sodium sulphate (3.5 g) for 38 hours at 37° C, the product (0.90 g), purified by ether precipitation and exhaustive dialysis contained 3.94 atoms of sulphur per 4 atoms of phosphorus (theoretical, calculated from the purine content of the original DNA, 3.85 atoms S per 4 atoms P); only 0.02 moles of adenine and 0.01 moles of guanine per 4 atoms of phosphorus remained and the pyrimidine content was virtually unchanged. The mercaptalation procedure rendered only 6.2% of the total phosphorus dialysable.

During this investigation, a method for the mercaptalation of herring roe DNA using ethyl mercaptan and concentrated hydrochloric acid was reported by LUCY AND KENT[7]. In our hands, however, this method did not give satisfactory results. Thymus DNA did not dissolve in the reagent but formed an insoluble gum. Upon shaking the reaction mixture for 16 hours at 0° C, only 50% of the theoretical amount of sulphur was introduced into the nucleic acid and already 15% of the phosphorus was dialysable; after 5 days shaking at 0° C over 90% of the material had become dialysable. It appeared, therefore, that this mercaptalating agent caused extensive degradation and so was not sufficiently reliable for investigations of the structure of thymus DNA.

The DNA treated with thioglycollic acid (TDNA) was readily degraded with alkali. After treatment at pH 12 at 37° C for 26 hours, 46% of the phosphorus was dialysable against dilute alkali (pH 12) and a further 50% against 2 *M* sodium chloride. It is interesting to note that CHARGAFF *et al.*[8] have reported the production of dialysable fragments by the alkaline degradation of apurinic acid, the material obtained by removing the purines from DNA at pH 1.6[9]. It would appear that these fragments might be somewhat similar to those obtained from our TDNA. In the latter case, however, there was the great advantage that the size of the fragments could be estimated from their S:P ratios.

The alkaline hydrolysate of TDNA was neutralised and the components separated by paper chromatography using various solvent systems and paper electrophoresis at various pH values. In this manner it was shown that there were present at least eleven components. One of these contained 1.95 moles of thymine to one of cytosine and 3.94 atoms of phosphorus to two atoms of sulphur. These results indicated that the substance was a trinucleotide linked to a mercaptalated 2-deoxy-D-ribosephosphate residue. The nucleotide sequence was, therefore, either cytosine-thymine-thymine or thymine-cytosine-thymine or possibly a mixture of the two. It appeared that, in this case, fission at both *a* and *b* could not have occurred as this would have produced a sulphur-free polynucleotide,

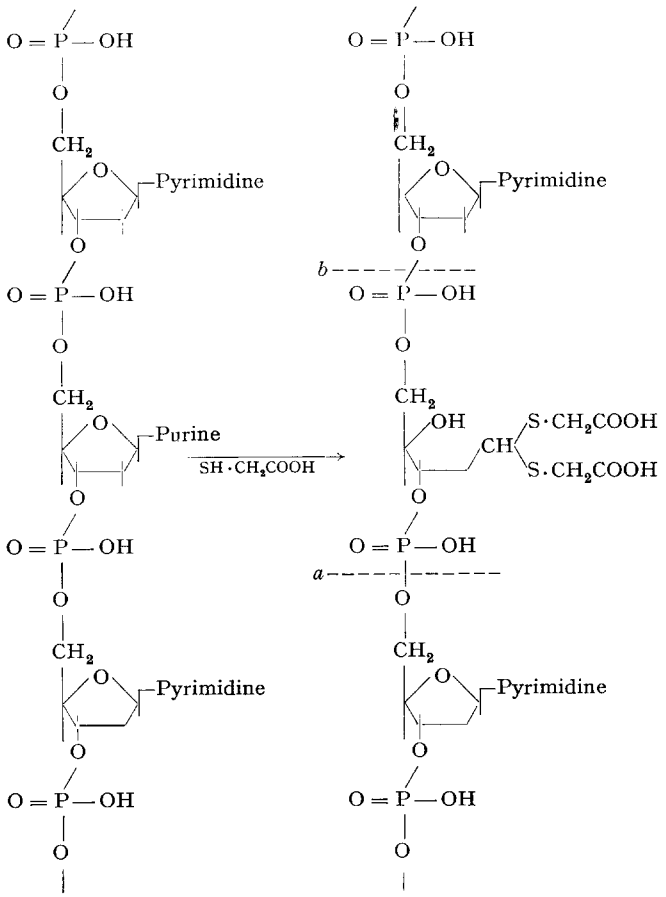

Fig. 1

but as yet no evidence has been obtained of the location of the 2-deoxy-D-ribosephosphate di(carboxymethylmercaptal) residues.

The material remaining in the dialysis sac after dialysis of the alkaline hydrolysate of TDNA against running tap water was found to run as a single zone both by paper chromatography and paper electrophoresis, although considerable streaking made it doubtful whether the substance was homogeneous. This material contained 1.48 moles of thymine to one of cytosine and 4.2 atoms of phosphorus to 2 atoms of sulphur. If alkaline degradation had occurred as in *a* or *b*, then it appeared that this material consisted mainly of trinucleotides linked to a terminal 2-deoxy-D-ribosephosphate di(carboxymethylmercaptal) residue. Work on the complete identification of these and other components is proceeding.

From the above results it was apparent that treatment with thioglycollic acid and subsequent alkaline degradation of DNA afforded a method for the determination of the sequence of the pyrimidine nucleotides. Although no products have been identified conclusively, there appeared to be present pyrimidine polynucleotides as complex as trinucleotides, thus indicating the presence in intact DNA of regions containing at least three pyrimidines linked together.

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THE INFLUENCE OF FEEDING OXYTHIAMINE ON THE THIAMINE PYROPHOSPHATE CONTENT OF SOME TISSUES OF THE PIGEON

by

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The recent publication of a paper by DE CARO, RINDI AND GRANA[1] on the metabolic effects of (neo)pyrithiamine* and the thiamine contents in the tissues of the rat prompts us to communicate the results of some experiments, carried out in 1952, regarding the effect of feeding oxythiamine on the thiamine pyrophosphate (TPP) content of tissues of the pigeon. It was originally intended to publish these results as part of more extensive investigations on the mode of action of thiamine antagonists, but considering the interest this subject evokes in various quarters, it seems preferable not to delay this information.

Since the discovery in 1943 by WOOLLEY AND WHITE[3] that small doses of pyrithiamine, given to mice maintained on a diet containing adequate amounts of thiamine, produced symptoms closely resembling those of thiamine deficiency, evidence has accumulated pointing to the fact that structural analogues of thiamine, notably pyrithiamine and oxythiamine[4,5], act as antagonists to the vitamin *in vivo*. Judging from comparative studies, performed by CERECEDO and collaborators[6,7], it would seem that pyrithiamine has a more pronounced antivitamin effect than oxythiamine, as the former calls forth symptoms of polyneuritis in mice while the latter fails to do so, although typical symptoms have been observed in chicks fed oxythiamine[8].

WOOLLEY[9] has compared the TPP contents of the livers of 2 groups of mice, both fed a highly purified ration supplemented with thiamine and one group receiving a single dose of 0.5 mg pyrithiamine. Surprisingly, TPP was not reduced in the livers of the latter group as compared to the former, although the animals had developed severe symptoms of deficiency when they were sacrificed. Now in a deficiency provoked by deprivation of thiamine there is always a marked decrease of TPP in all tissues[10]. Although the precise mode of action of the antivitamin is as yet unknown, it is to be expected that it will somehow interfere with the replacement of TPP in the appropriate enzymes and if catabolism exceeds replacement, TPP will gradually disappear.

These considerations induced us to investigate this point. For practical reasons[11] pigeons were used as test animals and oxythiamine—much more easily obtainable than pyrithiamine—as antagonist**.

In the first experiment 2 groups of 6 pigeons each received—by forced feeding—2 g casein and 18 g sucrose daily, supplemented with salt and vitamin mixture[12] and 100 γ thiamine daily. In addition one group received 1 mg oxythiamine daily. After 15 days the animals were sacrificed and TPP was determined manometrically[13] in extracts of liver, heart muscle (left ventricle), breast muscle (pectoralis major) and brain (cerebrum). At this time the animals in the oxythiamine group did not yet show any pronounced symptoms of thiamine deficiency. Two animals in the control group receiving thiamine only had died from other causes. The average values and the ranges found

* In accordance with WOOLLEY's suggestion[2] the pure pyrithiamine will be called pyrithiamine and not neopyrithiamine.

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