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# Further Studies on the Turnover of Polynucleotides in Regenerating Rat Liver

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A further study has been made on polynucleotide and protein turnover in regenerating rat liver, using $^{15}N$-labeled glycine. Liver tissue at 14, 18, 26, 37 and 126 h after hepatectomy was fractionated into nuclei and cytoplasm and the incorporation of glycine nitrogen into PNA and DNA purines and into total protein was determined. The purine bases from cytoplasm PNA were further degraded in order to analyze separately $^{15}N$ incorporation into the amino group, into $N_7$ of the ring, and into the pool of ring nitrogen atoms 1, 3 and 9.

The time curves of incorporation into both polynucleotides and protein were, for the most part, in agreement with those found in earlier work. Incorporation into cytoplasm PNA purines showed an early peak at 18 h after hepatectomy and a second one at 37 h. Nuclear PNA tended to reach maximum uptake around 26 h, somewhat later than the major peak for cytoplasmic PNA. The peak of incorporation into DNA was at 26—37 h. The curve for incorporation into protein showed two maxima, at 26 and 37 h, respectively.

The animals in this series showed appreciably greater liver regeneration than did those in the earlier study, in which the diet contained synthetic amino acids instead of casein. The absolute levels of incorporation were also higher than those in the previous study.

This study aimed at exploring possible explanations for the double peak found previously in the time curve of PNA incorporation. The two possibilities specifically investigated, cellular heterogeneity of the PNA and differences among the constituent nitrogen atoms of the purines, could not explain this double peak. Although the uptake into nuclear PNA was much greater than into cytoplasm PNA, both showed essentially the same time changes in incorporation. Likewise, the various nitrogen atoms of the purines followed time curves similar to those seen for the parent compounds. The high ratio of incorporation into guanine to that into adenine also carried over into all the constituent nitrogen atoms.

This study was undertaken in conjunction with another more intensive examination of the time curves of polynucleotide and protein turnover in regenerating liver, as indicated by the incorporation of $^{15}N$-glycine [1]. This

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previous study included time intervals very close together in the early period after hepatectomy, and the results revealed a somewhat surprising complexity in the time curves of incorporation for the purine bases of the nucleic acids. Of particular interest was a double maximum in the incorporation into the pentose nucleic acid (PNA) purines, the higher of the two peaks occurring at 14 h, and the second, lower one somewhat later. It was thought possible that this double maximum might be explained by cellular heterogeneity of the PNA, since the previous study had been made on total cell PNA, or by differences among the various nitrogen atoms of the purines, which had not been examined separately. The present study was undertaken to investigate these specific possibilities.

## EXPERIMENTAL

The study was again made on groups of animals at various stages of liver regeneration. The present study included fewer time points than did the previous study, and larger groups of animals were used. Five groups of adult albino rats weighing 180—200 g were subjected to partial hepatectomy [2] and the livers allowed to regenerate for 14, 18, 26, 37 and 126 h, respectively (time from operation to death). The groups were made up of 17 to 29 animals, depending upon the amount of liver tissue which could be expected for that particular regeneration stage.

During all but the longest regeneration period the animals were fed every four hours by stomach tube to insure adequate food intake. The diet was somewhat different from that used in the earlier study [1]. The one used previously was made up from synthetic amino acids and was to serve as a basis for further studies of amino acid deficient diets. Since preliminary work indicated no effect on liver growth or isotope uptake from such deficient diets, this rather elaborate diet was abandoned and replaced in the present study by one containing 18 % casein instead. This diet was given as a water suspension containing 10 g per 24 ml, and each rat received 4 ml of this suspension per 200 g (weight at time of hepatectomy) at each feeding. The animals undergoing 126 h of liver regeneration received the same diet in dry form rather than by stomach tube; they obtained and consumed 12 g per day.

An aqueous solution of labeled glycine (32.9 atom per cent excess $^{15}N$) was injected subcutaneously at a total dosage of 200 mg per 200 g (weight at time of injection). This was divided into two equal doses given 5 and 4 h, respectively, before death.

The regenerated livers from all animals were removed and weighed, and the average extent of regeneration calculated for each group, as described in previous work [1].

The livers for each group of animals were pooled, homogenized, and fractionated into nuclei and cytoplasm in citric acid, as in earlier work [2,3]. The nuclei were washed, disintegrated by vibration with glass beads in alcohol [4], and extracted with alcohol-ether to remove lipids [1]. The cytoplasm material was precipitated with alkali [3] and similarly extracted with alcohol-ether. From the dried tissue preparations mixed polynucleotides were then extracted by the method of Hammarsten [4], hydrolysed in alkali, and separated into PNA and desoxypentose nucleic acid (DNA) with lanthanum acetate [4].

The solutions of PNA nucleotides were hydrolyzed in 1 N hydrochloric acid at 100°C for 1 h, and the resulting mixture of purine bases and pyrimidine nucleotides was fractionated on Dowex 50, the pyrimidine components being eluted with 0.1 N acetic acid and the purine bases with 2 N hydrochloric acid [5]. Aliquots of each of the purines were purified by starch chromatography and the purity was checked and the $^{15}N$ content determined in the usual way [1]. This determination constituted the entire examination of the nuclear PNA purines. In the case of cytoplasm PNA, however, only small portions of the purines were used for this estimation of the isotope content in the total compound. The rest of each purine fraction was deaminated with sodium nitrite and hydrochloric acid, according to a modification of the methods of Strecker [6] and Kossel [7], under conditions found from preliminary experiments to give the best recovery of oxypurine in each case. Aliquots of the resulting hypoxanthine and xanthine were further purified by Dowex 50 and starch [8] chromatography in the same way as the amino purines and used for deter-

mination of $^{15}N$. Other aliquots of the oxypurines were evaporated to dryness at an alkaline pH to remove contaminating ammonia and further degraded by combustion in 6 N hydrochloric acid at 175°C for 24 h [9] to break the purine ring. This splits off the $N_7$ atom in the form of glycine, leaving a pool of ammonia from the nitrogen atoms 1, 3, and 9 [10,11,12]. The resulting glycine and ammonia were isolated on Dowex 50 [13,14] and $^{15}N$ content determined on each.

The lanthanum-DNA was decomposed with potassium carbonate and the DNA precipitated with acid alcohol [1,15], dried, and hydrolyzed with dry hydrochloric acid in methanol [16]. The purines were dissolved, freed of excess hydrochloric acid, and purified on starch for determination of $^{15}N$ content. The quantities of DNA purines were too low to allow sufficient material for any further degradation.

The protein residues remaining after the extraction of polynucleotides were treated as described in earlier work [1] to determine the content of $^{15}N$ in the total protein and, when possible, in the glycine constituent as well.

## RESULTS

In comparison with the previous series [1], these groups attained appreciably greater liver regeneration. The two earliest stages showed around 40—45 % (weight of regenerated liver as per cent of weight of original liver), in agreement with results found previously for these time intervals, but the regeneration increased to 66 % by 37 h (compared to around 51 % for this time interval in the previous study). This amount of regeneration is equivalent to that found in earlier series at 80 h. In the present series the regeneration then increased to 73 % by 126 h, giving a much less steep curve for the later time period.

Table 1 gives the figures for the $^{15}N$ content in the various purines and the constituent nitrogen atoms analyzed, as well as in the nuclear and cytoplasmic protein and the glycine constituent of the latter. In general the absolute figures for isotope incorporation were higher than in earlier series.

In the case of protein the levels of incorporation of isotope were approximately the same in both nuclei and cytoplasm. If one compares the time curve of protein or glycine uptake of tracer with that of the previous series, the peaks of incorporation can be seen to occur at about the same time in both cases. The highest figures for the time points included here are at 26 h for the nuclear protein and at 37 h for the total cytoplasmic protein as well as for its glycine constituent. The stages of 44—56 h are not represented in this series.

It is difficult to compare the absolute figures for the purines with those of the earlier series, since those for the nuclear PNA purines here are so much higher than those for the cytoplasm PNA compounds. However, in view of the complexities of nucleic acid turnover [1], such a comparison may be invalid for two series as different as these. If the present series is considered alone, it can be seen that cytoplasm PNA adenine showed two peaks of incorporation, one at 18 h, and a lower one at 37 h. This is in agreement with the earlier series, except that there the initial peak occurred at 14 rather than at 18 h. These time changes could be seen not only in the figures for the purine base itself but also in those for the hypoxanthine obtained by deamination, those for the ammonia nitrogen derived from nitrogen atoms 1, 3, and 9, and those for the $N_7$ atom isolated as glycine. The latter showed the highest content of $^{15}N$, representing the most direct incorporation of the precursor. It can be seen, then, that the various nitrogen atoms did not materially differ except

*Table 1.*

| Hours of regeneration | $^{15}N$ excess * 14 | 18 | 26 | 37 | 126 |
|---|---|---|---|---|---|
| Cytoplasmic PNA | | | | | |
| Adenine | 0.486 | 0.592 | 0.451 | 0.473 | 0.095 |
| Hypoxanthine | 0.614 | 0.678 | 0.504 | 0.546 | 0.097 |
| Amino-N | 0.000 | 0.250 | 0.239 | 0.183 | 0.088 |
| $N_7$-glycine | 1.65 | 1.80 | 1.32 | 1.43 | 0.198 |
| $N_{1,3,9}$-ammonia | 0.268 | 0.303 | 0.232 | 0.279 | 0.029 |
| Guanine | 1.51 | 1.73 | 1.66 | 1.30 | 0.233 |
| Xanthine | 1.64 | 1.88 | 1.74 | 1.40 | 0.227 |
| Amino-N | 0.990 | 1.11 | 1.33 | 0.896 | 0.260 |
| $N_7$-glycine | 4.26 | 5.00 | 4.50 | 3.55 | 0.573 |
| $N_{1,3,9}$-ammonia | 0.916 | 1.25 | 1.03 | 0.928 | 0.108 |
| Nuclear PNA | | | | | |
| Adenine | 1.89 | 2.33 | 2.26 | 2.15 | 0.568 |
| Guanine | 4.32 | 6.34 | 7.14 | 5.52 | 1.48 |
| DNA | | | | | |
| Adenine | 0.040 | 0.043 | 0.286 | 0.288 | 0.041 |
| Guanine | 0.209 | 0.297 | 1.09 | 0.787 | 0.135 |
| Cytoplasmic protein | | | | | |
| Total | 0.734 | 0.835 | 1.10 | 1.15 | 0.754 |
| Glycine | 5.05 | 5.54 | 7.03 | 7.16 | 4.50 |
| Nuclear protein | | | | | |
| Total | 0.798 | 0.679 | 1.17 | 1.14 | 0.726 |

* The values are calculated on the basis of 100 % $^{15}N$ in the administered glycine.

in the level of incorporation; their changes of incorporation with time were essentially the same. The time curve for nuclear PNA adenine also showed an early maximum at 18 h, with the level falling off slowly after that time. This is then not greatly different from the pattern for cytoplasmic PNA adenine.

A comparison of these figures with those for the uptake into guanine shows that, as previously observed [1,17], the incorporation of glycine-$^{15}N$ into guanine was considerably greater than into adenine. From the figures for the degraded purines of cytoplasmic PNA, it can be seen that this difference between guanine and adenine uptake carried over into all the constituent nitrogen atoms and was not due to only one particular part of the respective molecules. The figures for the various time stages showed that, as in the previous series, the two purines did not follow identical time curves of incorporation, although the similarities between the two bases were somewhat greater in the present series. Cytoplasmic PNA guanine again showed an early peak at 18 h, with the level of incorporation falling off rather slowly after that time. Here, too, the same changes with time could be seen in the figures for the oxypurine, the $N_{1,3,9}$ ammonia, and the $N_7$ glycine, so that there were no real differences among the various nitrogen atoms in the time curve of incorporation. The nuclear PNA guanine also showed only one peak, which occurred, however, at 26 h rather than at 18 h.

The incorporation into the DNA purines was, as in other studies with glycine, less than into those of PNA, although of the same order of magnitude. The time curves of incorporation showed single peaks. There was a high maxi-

mum for guanine at 26 h, with the level decreasing somewhat slowly with time, while adenine showed a broad maximum between 26 and 37 h. Thus, the maximum incorporation into DNA occurred considerably later than did that for PNA; the figures here serve to emphasize the peak found for DNA at 26—32 h in the previous series.

## DISCUSSION

The difference between this series and the earlier one in amount of liver regeneration shown by the animals was somewhat unexpected. This difference may have been a result of the change from the synthetic amino acid diet to the casein diet. Other results in this laboratory [18] confirm more extensive liver regeneration on the casein diet than on the other one, and a casein diet has been found by others to be conducive to liver growth [19,20].

It is hard to know what effect more rapid regeneration may have had on the turnover picture, but the higher incorporation figures, compared with the earlier series, may well have been due to this greater extent of regeneration for all time points. Certainly, in view of this difference, it would seem to be somewhat hazardous to compare any absolute turnover figures for the two series.

With regard to the time curves, the two points of maximum protein turnover (26 h for nuclear protein and 37 h for cytoplasmic protein) confirm earlier findings [1,21] within the limitations of the time points included in the present series. In contrast, the time curves of nucleic acid incorporation for this series are somewhat different from those for the previous one. One major difference is the location of the inital PNA peak at 18 instead of at 14 h. It is possible that this delay in PNA uptake could have been due to the difference in diet; the casein diet could conceivably have been more slowly metabolized, which might have affected the incorporation during early stages of regeneration. However, the turnover picture for both PNA and DNA showed essentially the same changes with time as that found previously. Thus, the initial high peak of incorporation into PNA occurred considerably ahead of any other maximum, and there was a second increase in PNA uptake somewhat later; the peak of DNA incorporation of isotope also occurred at a later time. It can be seen, then, from these results that the specific possible explanations for heterogeneity of PNA turnover investigated in these experiments could not explain the time changes. Thus, when the cytoplasmic PNA purines were degraded and individual nitrogen atoms analyzed, the changes of isotope incorporation with time were found to be very nearly the same for all the various atoms. It is apparent, therefore, that the two peaks in the PNA uptake of isotope are not due to differences among these atoms. Especially in the case of adenine, the double peak could again be seen in this series, and the figures for the degradation products showed that the same time curve carried over into all the constituent nitrogen atoms.

It has been noted in previous work that glycine-$^{15}$N incorporation is greater into nucleic acid guanine than into adenine *cf.*[1] and this was confirmed by the present results. The figures here for the purine degradation products showed that the incorporation into xanthine was similarly high compared with that for hypoxanthine; the high ratio found in these experiments is, therefore, not

due to a difference in the metabolic activity of the amino group, which appears to be approximately the same for both purine bases. In fact, this high ratio also carried over into all the nitrogen atoms of the oxypurines, although it varied somewhat with time in each case. From these data adenine is not an intermediate in the synthesis of guanine from glycine, a finding supported by recent results of *in vitro* experiments [22-25]. Data on adenine incorporation, indicating an adenine to guanine conversion, merely reflect the probability that in this case the biosynthesis follows different pathways.

The double peak in PNA incorporation of isotope was also considered to be, possibly, an effect of the known cellular heterogeneity of PNA [2,3,17,26-34]. However, from these data, differences between cytoplasmic and nuclear PNA would seem to be unable to account for this double peak, since both showed essentially the same time changes in incorporation. In the case of adenine, a double maximum was apparent in the figures for cytoplasmic PNA alone, and the peak for nuclear PNA coincided with the main peak for cytoplasmic PNA. In the case of guanine, the peak for nuclear PNA occurred somewhat later than that for cytoplasmic PNA, and this difference may have, to some extent, contributed to the double maximum observed for guanine in the previous series. However, this contribution must have been small because of the great difference in the amounts of the two fractions, and in view of the slow decrease in cytoplasmic PNA incorporation here, the cytoplasmic PNA would appear to have been the major contributor to the later peak as well as to the first one. These conclusions do not, of course, eliminate the possibility of PNA heterogeneity of some other kind as an explanation of the turnover curve.

The absolute figures for incorporation into nuclear material, compared with cytoplasm, were very high for the PNA purines, in contrast with those for protein, which were approximately, the same in both cases. This has been found in work with various precursors [2,3,26-31], and the possible significance of the finding for the theory of a function of PNA in protein synthesis has been discussed [27]. However, although this nuclear PNA/cytoplasmic PNA ratio remained high throughout the regeneration time curve, the maximum incorporation by nuclear PNA occurred at a later period of regeneration than that by cytoplasm PNA. As pointed out above, this was especially obvious in the guanine figures. This finding may be of relevance in elaborating the relationship between nuclear and cytoplasmic PNA.

This later maximum of nuclear PNA bears upon the cytological findings in the previous paper [1]. In that study, the maximum of nucleolar size was observed around 10—15 h, which coincided rather well with the initial peak in PNA incorporation at 14 h. A correlation of the two is attractive but, on the basis of the present data, would appear to be erroneous, since the PNA of the nuclei is apparently much less important than the cytoplasmic PNA in this first PNA peak. This makes it impossible for the part of the nuclear PNA located in the nucleolus to account for the peak, and the coincidence of the two is, therefore, probably a chance one.

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