ACTA CHEMICA SCANDINAVICA 5 (1951) 431—444

Turnover Rates During Formation of Proteins and Polynucleotides in Regenerating Tissues

N. A. ELIASSON, E. HAMMARSTEN, P. REICHARD and S. ÅQVIST

Biochemical Department, Karolinska Institutet, Stockholm, Sweden

BO THORELL

Department of Cell Research, Karolinska Institutet, Stockholm, Sweden

G. EHRENSVÄRD

Wenner-Gren Institute, University of Stockholm, Stockholm, Sweden

The simultaneous appearance of protein and nucleic acid in growing cells was discovered by Miescher [1]. Kossel [2] later contended that a simultaneous formation of basic proteins and polynucleotides is a fundamental reaction in cell nuclei, connected with the processes of cell division, fertilization and inheritance.

More recently several investigators, for example Masing, Fridericia, Brachet, Caspersson *et al.*[3-6], have demonstrated the fact that an increase in the amount of polynucleotides per weight or volume of cell occurs during early stages of growth. However, there is very little information about the appearance of the proteins that are synthesized during growth and formed apparently at about the same time as the polynucleotides.

The authors of the present paper wanted to investigate a possible correlation between the turnover and synthesis of protein and that of polynucleotides during growth. Glycine marked with N^{15} was used as tracer-precursor in these experiments. The rate of protein turnover was measured by the concentration of isotope in the glycine incorporated into the proteins at different stages of regeneration of rat liver. In the same experiment the polynucleotides were degraded and the rate of their turnover measured by the isotope content in the nitrogenous bases.

Hen bone marrow was also used in some experiments in order to study the metabolic changes during growth and differentiation of the blood cells. Re-

sults of these experiments will be mentioned here only in so far as they disclose a certain relationship between the isotope content in amino acids and purine bases.

EXPERIMENTAL

Regeneration of liver

Albino rats of 180 to 200 g were used. The animals were obtained from various sources and constancy of strain could not be controlled. This unfortunate fact may have introduced biological variations. To minimize the effect of this we have pooled the livers from 20—40 rats for the analysis at each regeneration stage. This has also made it possible to include in the analytical plan the small fraction of PNA in the nuclei.

The animals had free access to a mixed diet of milk, bread and oats, both before and after the operation. Their consumption of food was only roughly estimated. The hepatectomized animals began to drink milk about half an hour after the operation but they consumed less food than animals which had not undergone hepatectomy.

Partial hepatectomy was performed in the same way as in earlier work [7]. Isotopic glycine (31 atom per cent excess N^{15}) was injected subcutanously at a level of 100 mg of glycine per 100 g of the body weight at the time of operation. Injections were made every two hours, four times in all, during the last eight hours before the animals were sacrificed. The rats were killed two hours after the last injection of isotopic glycine.

Five groups of animals were used with twenty to forty rats in each depending on the stage of regeneration. After preliminary experiments with smaller groups the following regeneration times (hours of life from operation to death) were chosen: 11, 26, 32, 56 and 170.

The only intentional difference between these groups is the stage of regeneration. Each group received the same amount of isotope during the same period before death.

Table 1. Regeneration at different times after partial hepatectomy.

No. of animals	Regeneration time in hours	Regeneration in per cent and standard deviation
40	11	2 ± 13
30	18	32 ± 19
30	26	50 ± 13
25	24	34 ± 19
25	32	34 ± 14
20	48	102 ± 32
20	56	65 ± 19
20	162	131 ± 71
20	170	142 ± 44

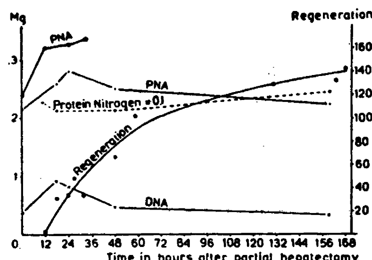

Fig. 1. Mg per 100 mg dry weight of liver. PNA and DNA as phosphorus. Protein as nitrogen. Regeneration as increase in per cent of the weight of the remaining lobe after partia l hepatectomy.

The excised portions of the liver were always weighed. In a separate experiment on 25 rats both the excised and the remaining portions of the liver were weighed; in this experiment the weight of the remaining part was found to be 57.8 % of the weight of the excised part with a standard deviation of 5.7. This figure has been used to calculate from the weight of the excised liver in each case, the weight of the liver remaining after partial hepatectomy at regeneration time zero. From this figure and from the final weight of the liver after regeneration, the amount of regeneration can be calculated in grams of wet weight. In this paper the extent of regeneration is defined as the increase in wet weight of the remaining liver lobe, expressed as per cent increase of its calculated weight at the time of operation.

At each regeneration stage samples of liver tissue were removed for cytological investigations.

In separate experiments the extent of regeneration was determined for regeneration times eight hours less than the regeneration times in the actual isotope experiments (3, 18, 24, 48 and 162 hours). The differences between these figures and those found for regeneration in the isotope experiments were taken as indicative of the extent of regeneration during the last eight hours, *viz.* the period when isotopic glycine was available in the body. In 5 to 10 % of the cases the liver showed extremes of very low or excessive growth. These extremes are included. The variations are too large to allow a determination of the point of maximum growth during the eight hour periods of tracer turnover (Table 1).

The separation of cell nuclei and cytoplasm, the preparations of protein and polynucleotide fractions and the degradation of the latter were carried out by methods described in earlier publications [8]. The purification of the liver cell nuclei was controlled by phase contrast microscopy.

Regeneration of blood cells in the hen was brought about by phenylhydrazine hemolysis. Fifty mg of phenylhydrazine per kg of body weight were injected subcutaneously. After 54—60 hours the frequency of mitosis in the bone marrow had reached its highest value and the relative number of immature cells in the blood stream was at its maximum. At this point intraperitoneal injections of N^{15}-labeled glycine were begun; three injections were made at 6 hours intervals, the total amount injected being 2.0 g per kilogram of body weight. The hen was sacrificed by exsanguination after a further 6 hours period (72 hours from the time of hemolysis). Further particulars of this regeneration and the results on the formation of cellular substances during the blood cell production will be published elsewhere.

Table 2. The isotope content in glycine + serine compared with the sum of that in fourteen of the other amino acids (incl. humin and ammonia). The values are given in atom per cent N^{15} calculated on basis of 100 per cent in administered glycine.

Hen no.	Organ	I Glycine + Serine	II Amino acids	I/II
1	Bone marrow normal hen	19.8	11.6	1.71
3	Bone marrow phenylhydrazine 4 g glycine/kg	43.4	26.4	1.64
1	Liver normal hen	12.9	9.6	1.35
2	Liver phenylhydrazine 2 g glycine/kg	14.2	11.4	1.25
3	Liver	22.2	17.2	1.29

Regenerating liver from rat.

Regeneration time in hours	Glycine + Serine I	Amino acids II	I/II
11	11.13	5.92	1.88
26	16.06	7.17	2.24
32	14.06	10.17	1.38
56	18.22	11.92	1.53
170	14.36	8.54	1.68

Glycine and the other amino acids were separated from hydrolysates of the liver and bone marrow proteins by starch chromatography according to the method of Moore and Stein [9] but on an enlarged scale to permit the isolation of milligram amounts of each amino acid. Details of this separation will form the subject of an other paper [10].

RESULTS

A general picture of the processes of polynucleotide and protein synthesis during different stages of regeneration is given by Fig. 1. The amounts of polynucleotide phosphorus per gram of dry liver tissue were determined by the

Table 3. Correlation of isotope contents of glycine and serine to adenine and guanine in experiments on hens with glycine-N^{15}. The values are given as atom per cent N^{15} calculated on basis of 100 per cent in administered glycine.

Hen no.	Organ	Glycine/ Adenine	Glycine/ Guanine	Serine/Adenine	Serine/Guanine
1	Bone marrow normal hen	$\frac{12.85}{5.17} = 2.49$	$\frac{12.85}{5.01} = 2.56$	$\frac{6.97}{5.17} = 1.35$	$\frac{6.97}{5.01} = 1.39$
2	Bone marrow phenylhydrazine	$\frac{21.24}{6.10} = 3.48$	$\frac{21.24}{7.50} = 2.85$	$\frac{11.59}{6.10} = 1.90$	$\frac{11.59}{7.50} = 1.55$
3	Bone marrow phenylhydrazine, 4 g glycine/kg	$\frac{26.55}{8.56} = 3.10$	$\frac{26.55}{9.68} = 2.74$	$\frac{16.84}{8.56} = 1.97$	$\frac{16.34}{9.38} = 1.74$
1	Liver	$\frac{7.34}{2.66} = 2.76$	$\frac{7.34}{3.65} = 2.01$	$\frac{5.52}{2.66} = 2.07$	$\frac{5.52}{3.65} = 1.51$
2	Liver	$\frac{7.61}{2.11} = 3.61$	$\frac{7.61}{3.08} = 2.47$	$\frac{6.54}{2.11} = 3.10$	$\frac{6.54}{3.08} = 2.12$
3	Liver	$\frac{12.38}{5.04} = 2.44$	$\frac{12.28}{5.48} = 2.24$	$\frac{9.89}{5.04} = 1.96$	$\frac{9.89}{5.48} = 1.80$

method of Hammarsten. The levels of both PNA and DNA show a maximum during early stages of regeneration. When the amount of trichloroacetic-acid (TCA)-insoluble nitrogen was determined no definite changes was observed.

Distribution of isotope in the proteins. The isotope content of the amino acids isolated from the proteins was determined. Table 2 shows a survey of the distribution of N^{15} from administered glycine in the other amino acids. The N^{15} content of isolated glycine is about ten times that of any one of the other amino acids into which N^{15} has presumably been introduced mainly by transamination reactions. The one exception is serine which is known to be synthesized directly from glycine.

When the total amounts of isotopic nitrogen in the amino acids were calculated from the isotope contents and the approximate amounts of amino

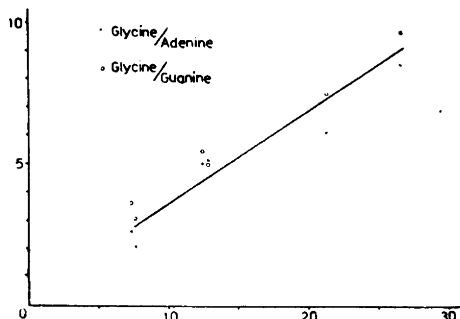

Fig. 2.
Ordinate: *N^{15} content in adenine and guanine.*
Abscissa: *N^{15} content in glycine.*
All values calculated on basis of 100 per cent in administered glycine.

acids, it was found that glycine contained 40 per cent and serine 20 per cent of the total isotopic nitrogen in the proteins from rat cytoplasm.

Experiments on hens. Table 3 and Fig. 2 give various values for ratios between N^{15} incorporation into the two amino acids glycine and serine (from proteins of different organs) on the one hand and the N^{15} incorporation into the polynucleotide purines guanine and adenine on the other. The specific ratios chosen were glycine / adenine, glycine / guanine, serine / adenine and serine / guanine. The figures demonstrate that when the amino acid shows an increase in N^{15} the purine compound does likewise.

Liver regeneration. Table 4 and Figs. 3, 4 and 5 summarize our findings in the experiments on regenerating liver. In the tables the ratio $\frac{E_{max}}{\gamma N/ml}$ is a test of purity with respect to freedom from foreign nitrogen [7]. Some of the values have been determined in duplicate (for separate animal groups) and in these cases the duplicate values have been recorded in the tables 3, 4 and 5.

It can be seen from the figures that the rate of synthesis of purines has a maximum at the 26-hour point (time of isotope administration 18—26 hours after hepatectomy). This maximum is especially high in the case of guanine from cell nuclei PNA [7]. The position of the maximum is the same for all the purines. We do not want to state that this would hold true if more frequent regeneration stages were measured in this region.

The curve for glycine represents the rate of synthesis of proteins; here the values are not as much above the "normal" incorporation of N^{15} in non-regenerating liver as in the case of the purines. The figures show a maximum situated beyond the purine peak at about the 56 hour point. The figures for N^{15} incorporation into the TCA-insoluble fractions from cytoplasm show the same maximum (See Table 5).

Table 4. Injection of glycine-N^{15} in rat. Regenerating liver.

Isolated substances	Cell nuclei				Cytoplasm	
	PNA Atom per cent excess N^{15}	E_{max} / μg N/ml	DNA Atom per cent excess N^{15}	E_{max} / μg N/ml	PNA Atom per cent excess N^{15}	E_{max} / μg N/ml
	Regeneration during 11 hours					
Guanine	.782	.160	.053	.159	.283	.162
					.302	.155
Adenine	.488	.172	.006	.180	.165	.180
					.197	.175
Cytidine	.342	.285			.081	.287
	.476	.292				
Uridine	.534	.325				
	.589	.341			.155	.326
Thymine			.030	.270		
			.014	.264		
Cytosine			.020	—		
			.012	.223		
	Regeneration during 26 hours					
Guanine	1.722	.162	.641	.158	.637	.167
Adenine	(.868)	—	.209	.178	.251	.183
Cytidine	.503	.287			.224	.294
Uridine	.698	.340			.254	.332
Thymine			.085	.274		
Cytosine			—	—		
	Regeneration during 32 hours					
Guanine	1.441	.162	.171	.165	.587	.165
Adenine	.330	.178	.099	.169	.214	.180
Cytidine	.541	.291			.205	.308
Uridine	.665	.322			.228	.332
Thymine			.053	.264		
Cytosine			.078	.218		
	Regeneration during 56 hours					
Guanine	.833	.161	.146	.158	.279	.164
Adenine	.380	.180	.089	.172	.141	.182
Cytidine	.421	.288			.214	.291
Uridine	—				.143	.328

Table 4 continued.

	Regeneration during 170 hours					
Guanine	.225	.164	.071	.159	.086	—
	.344	.160			.083	.173
Adenine	.213	.174	.023	.176	.061	—
	.132	.175	.024	.179	.044	.183
Cytidine	.180	.290			.103	.293
					.053	.296
Uridine	.076	.332			.061	.329
					.115	.336
Thymine			.031	—		
			.012	.268		
Cytosine			.015	.231		

The curves for the pyrimidines are between glycine and the purines. For reasons outlined in the discussion we will not try to evaluate the pyrimidine values as a measure of polynucleotide synthesis.

The cytological findings are recorded in Fig. 6. This shows the changes in the optical cross-section area of the liver cell parts during regeneration. The measurements were made on 5μ sections stained with Ehrlich hematoxylin-eosin. The sections were made from pooled samples of the same livers as were used in the isotope experiments. The ratios between the nucleolar, nuclear and cytoplasmic areas were obtained by Chalkley's method for recording ratios of points indicated by ocular pointers in the plane of the microscope image [11,12]. Optics: Objective apochromat 90 x, num. ap. 1.35, ocular comp. 10 x. For each regeneration time a total of 2 500—3 000 loci were recorded.

The nuclear diameters were measured in 150—200 liver parenchymal cells at each regeneration stage, using an eye piece screw micrometer. By assuming

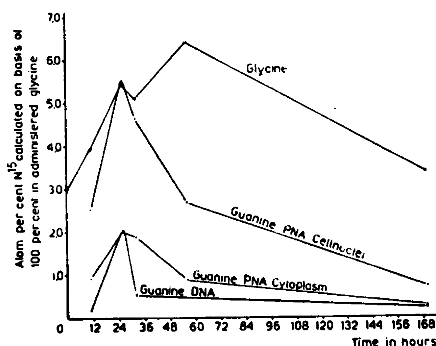

Fig. 3. The contents of N^{15} are calculated on basis of 100 per cent in administered glycine. The values for glycine (Table 5) are multiplied with 2/3 to reduce the height of the glycine curve.

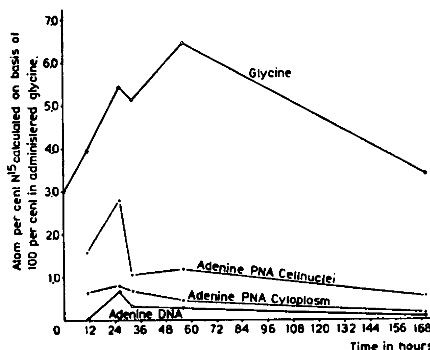

Fig. 4. The contents of N^{15} are calculated on basis of 100 per cent in administered glycine. The values for glycine (Table 5) are multiplied with 2/3 to reduce the height of the glycine curve.

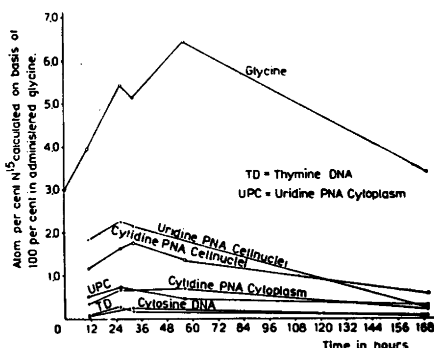

Fig. 5. The contents of N^{15} are calculated on basis of 100 per cent in administered glycine. The values for glycine (Table 5) are multiplied with 2/3 to reduce the height of the glycine curve.

Table 5. Injection of glycine N^{15} in rat. Regenerating liver.

Regeneration time in hours	Atom per cent excess N^{15}. The values are given in atom per cent N^{15} calculated on basis of 100 per cent in administered glycine.			
	Glycine	Serine	Hydrolysate of total protein	
			Cell nuclei	Cytoplasm
0	4.53	3.97	.741	.753
11	5.93	5.20	.757	.725
26	8.14	7.92	1.40	1.19
32	7.68	6.38	1.19	1.17
56	9.66	8.56	1.30	1.38
170	5.15	6.11	.712	.708

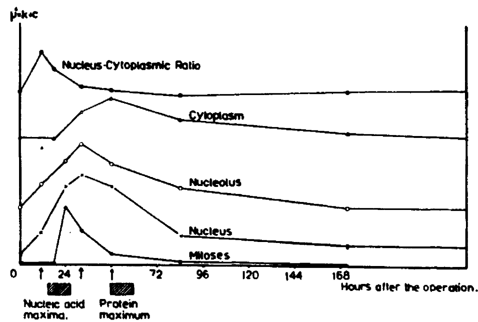

Fig. 6. Cytological changes during liver regeneration. To make data easily understood each curve is multiplied by an arbitrary factor and presented separately by the addition of a constant. The maximum values, expressed as percentage increase of the normal liver cell constituents, are as follows: Nucleolus 260, nucleus 160, cytoplasm 145 and nucleus-cytoplasmic ratio 130. The mitosis frequencies increased from less than 0.05 % in normal liver to 10 ± 0.32 % at 24 hours regeneration.

that the nuclei are spherical, the relative changes in the optical cross-section area of the cell parts can be easily calculated.

The mitosis frequencies were measured as a Poisson distribution using the above described optics. A total of 2 000 cells were counted at each regeneration stage.

The course of the cytological changes during liver regeneration, as illustrated in Fig. 6, does on the whole agree with the rather scanty earlier data [13]. It is interesting to note the coincidence of the maximum rate of polynucleotide synthesis with the maximum of mitosis frequency and with the maximum

Table 6.

Isotope	C^{13} Atom per cent excess	C^{14} Counts/min	N^{15} Atom per cent excess
I. Administered glycine	13.43	30 336	31.0
II. Isolated glycine	0.945	1 692	2.38
Excess in isolated glycine as % of administered glycine (Specific activity)	7.0	5.6	7.7

size of nuclei and nucleoli. Also it can be seen that during the period of maximum protein synthesis at regeneration times of 48—56 hours the above mentioned cytological quantities have already diminished. Further data on the liver cytology in relation to turnover values will be published elsewhere.

DISCUSSION

The investigation of the relationship between the turnover rates of two different cellular substances will naturally be dependent to a large extent on the precursor used. The ideal precursor should be directly incorporated into both of the actual substances without any intermediates, a situation which probably can be realized only in a very few cases.

In order to investigate to what degree glycine has been incorporated as an intact molecule, thrice-marked glycine was used in some experiments. The glycine contained C^{13} in the methyl-, C^{14} in the carboxyl- and N^{15} in the amino-group. The excess of isotopes was determined in a sample of glycine isolated from proteins.

The figures in Table 6 are the direct analytical values measured as excess of total carbon. No attempts have been made to degrade the glycine and determine the isotope figures in each carbon atom. The values show that the ratio between the excess of C^{13} and that of N^{15} is nearly the same in the glycine isolated from proteins as in the administered compound. The values for C^{14} show that the glycine incorporated into the protein molecule has undergone slight rearrangement involving rupture of carbon to carboxyl group. The analyses give no information, however, about a possible C^{13} content in the carboxyl group.

In any case these analyses show that the administered glycine has been directly incorporated into the proteins with only some slight intermediate turnover of the carboxyl group. The glycine can be regarded as fairly well distributed in the protein molecule, and we think that the isotope content of the glycine isolated from the proteins can be used as a measure of the protein turnover.

In the polynucleotides the incorporated glycine is probably situated mainly in position 4,5 and 7 of the purines. A great part of the glycine thus can be regarded as being a direct precursor for the purines. In the case of the pyrimidines, however, we know very little about the incorporation of glycine.

When glycine-N^{15} was used as tracer-precursor in rapidly regenerating bone marrow and non-regenerating liver in the same hen, a correlation could be observed between the isotope contents in guanine and adenine from the polynucleotides and the isotope contents in glycine from the proteins (Table

3, Fig. 2). Certainly the above-mentioned direct incorporation of glycine into both the purines and the proteins plays an important role in this correlation.

In spite of the wide deviations from a mean value we think that this correlation is not accidental but indicates a general tendency of simultaneous metabolic activity of polynucleotides and proteins. This connection might possibly signify a functional interaction of polynucleotide and protein turnover and should be considered along with the turnover rates found in the experiments on regenerating rat liver.

In these experiments the values in Table 4 and Figs. 3—5 show that the maximum turnover rates for all nitrogenous compounds in PNA in cell nuclei and in cytoplasm and in DNA appeared at about 30 hours after partial hepatectomy. The greatest increase in the amount of PNA and DNA per dry weight liver tissue appeared at the same time (Fig. 1), demonstrating the coincidence of high turnover rates with an increase of polynucleotides per dry weight of tissue.

The maximum turnover rate of glycine in the proteins appeared at approximately 30 hours later (60 hours after partial hepatectomy). All of the other amino acids also had a maximum at this point, indicating a common maximum of transamination. The regeneration curve and the curve for protein nitrogen (Fig. 1) demonstrate the well known fact that a protein formation takes place during regeneration. As we failed to determine the point of maximum regeneration during the eight-hour periods of isotope turnover, we can only state that glycine incorporation occured at a time when the total amount of proteins was increasing. We think that in a situation where a very rapid formation of proteins occurs this protein synthesis may have to be preceded by a rapid turnover and synthesis of polynucleotides. This would not necessarily exclude the possibility of some protein synthesis during stages of comparatively low turnover and synthesis of the polynucleotides if the concentration of polynucleotides was already sufficiently high.

The amount of food consumed during the time of regeneration may well influence the position of turnover maximum as indicated by some new experiments we have made. We are therefore continuing the investigation with controlled feeding starting four hours after the partial hepatectomy.

It has long been assumed that there is some fundamentally important interaction between polynucleotides in the cell structures and the synthesis of proteins. There seems to be no doubt about the rather sudden increase in the amounts of polynucleotides in phases of high growth activity. Chemical analysis — like those in the present work — and studies of absorption in the ultraviolet region of the spectrum, have clearly demonstrated the rapidity with which these changes take place [6]. The relationship of the protein synthesis to

these changes has, however, remained obscure. Attempts to correlate the rate of growth with the cellular polynucleotides by means of UV-absorption measurements, although very informative about the polynucleotide changes during different physiological conditions of the cell, cannot be considered conclusive with respect to the protein synthesis.

As has been pointed out, there is ample evidence that polynucleotides are synthetisized during stages of growth when proteins are formed. This was first shown for semen by Miescher in 1874, for embryonic liver by Masing in 1911 and more recently for rat liver regenerating after partial hepatectomy by Brues *et al.*, Novikoff and Potter [14-16] and others. Our experiments show a connection during growth between the turnover rates of polynucleotides and proteins. Furthermore they give a time schedule for these two synthetic processes. The linking mechanism, however, is quite unknown.

SUMMARY

Glycine marked with N^{15} was used as tracer-precursor in rapidly regenerating bone marrow and non-regenerating liver in the hen. A correlation could be demonstrated between the isotope contents in the glycine isolated from the proteins and the isotope contents in the purine bases from the polynucleotides.

Rat liver in different stages of regeneration after partial hepatectomy was analyzed for the content of N^{15} in the amino acids from the proteins and in the nitrogenous bases from the polynucleotides in cell nuclei and cytoplasm. Glycine labeled with N^{15} was again used as isotope precursor. Under the conditions of these experiments the maximum rate of polynucleotide turnover occured at about 30 hours after the operation and the maximum rate of protein turnover at about 60 hours.

REFERENCES

1. Miescher, F. *Arch. exptl. Pathol.* **37** (1896) 100.
2. Kossel, A. *The protamines and histones*, London-New York (1928).
3. Masing, E. *Z. Physiol. Chem.* **75** (1911) 135.
4. Fridericia, X. *Scand. Arch. Physiol.* **26** (1912) 1.
5. Brachet, J. *Acta Biol. Belg.* **2** (1942) 13, *Sympos. of the Soc. of exp. Biol. Cambridge* (1947) 207.
6. Caspersson, T. *Naturwissenschaften* **29** (1941) 33, *Sympos. of the Soc. for exp. Biol. Cambridge* (1947) 127.
7. Bergstrand, A., Eliasson, N. A., Hammarsten, E., Norberg, B., Reichard, P., Ubisch, v. H. *Cold Spring Harbor Symposia* **XIII** (1948) 22.

8. Hammarsten, E. *Acta Med. Scand.* **196** (1947) 634.
Edman, P., Hammarsten, E., Löw, B., Reichard, P. *J. Biol. Chem.* **178** (1949) 395.
Reichard, P. *J. Biol. Chem.* **179** (1949) 763.
Reichard, P. *J. Biol. Chem.* **179** (1949) 773.
Reichard, P. *Acta Chem. Scand.* **3** (1949) 422.
9. Stein, W. H., and Moore, S. *J. Biol. Chem.* **178** (1949) 79.
10. Åqvist, S. Unpublished.
11. Chalkley, H. W. *J. Nat. Cancer Inst.* **4** (1943) 47.
12. Lagerstedt, S. *Acta Anat.* Suppl. IX (1949).
13. Stowell, R. E. *Arch. Path.* **46** (1948) 164.
14. Brues, A. M., Drury, D. R., and Brues, M. C. *Arch. Path.* **22** (1936) 658.
15. Brues, A. M., Tracy, M. M., and Cohn, W. E. *J. Biol. Chem.* **155** (1944) 619.
16. Novikoff, H. B., Potter, R. *J. Biol. Chem.* **173** (1948) 233.

Received December 20, 1950.