Reports

Turnover of Young and Old Serum Proteins

Up to now it remains unresolved whether the breakdown of serum protein molecules depends on their age, the oldest protein molecules breaking down first, or whether the protein molecules break down randomly. In experiments in which radioactive tracers are involved, it is assumed inherently that newly formed serum protein molecules and older molecules of the same type are identical and that they are metabolized at the same rate. The validity of this assumption has not yet been proved. The experiments described in this report had the purpose of determining whether newly formed and older rat serum proteins, after injection into young rats, have a similar turnover time or whether the older molecules are broken down at a higher rate (*1*).

A yeast protein hydrolyzate containing S^{35}-amino acids was prepared as previously described (*2*). Four Sprague-Dawley rats, weighing approximately 100 g each, from four separate litters, were injected intraperitoneally with 2.4×10^9 count/min of the amino acid hydrolyzate. Eight hours later the blood was withdrawn from these donor rats by heart puncture. This blood was pooled and allowed to clot, and the serum was removed. We designate the labeled serum proteins in this preparation as "young" proteins. Similarly, four other donor rats from four different litters were injected with 9×10^9 count/min of the amino acid hydrolyzate. One of the rats died after 3.5 days (possibly of radiation sickness). The remaining three rats were bled by heart puncture 93.5 hours after injection. The labeled serum proteins in this pooled preparation are designated as "old" proteins. The bulk of the globulins of the "young" and "old" sera was removed by 50-percent saturation with ammonium sulfate; the albumin fraction was salted out by saturation of the supernatant solution with ammonium sulfate. The precipitate was dissolved in 45 ml of water, brought to *p*H 8, mixed with 18 mg of cysteine, and kept in the cold room for 2 hours. It was then dialyzed against water. Paper electrophoresis showed that 72 percent of this albumin fraction consisted of albumin, and 11, 13, and 4 percent of α-, β-, and γ-globulin, respectively.

The radioactive "young" albumins were injected via the femoral vein into four litters of Sprague-Dawley rats, each litter consisting of seven or eight animals. The dose injected per recipient rat was 0.84 mg (260,000 count/min) in 0.2 ml. Fifteen days later four other litters of recipient rats were injected, each animal receiving 1.1 mg (130,000 count/min) of the "old" serum protein in 0.4 ml. During this period of 15 days, the weight of the second group of animals increased so that the average weight at death was 137 g for the first group and 203 g for the second group of animals.

Rats (one from each litter) were exsanguinated by heart puncture 1, 2, 3, 4, 5 (or 5.8), 8, and 9 days after injection, and their livers were removed. The blood was allowed to clot; the serum was then removed and the bulk of the globulins was salted out by half-saturation with ammonium sulfate. The supernatant albumin fraction was dialyzed against water and subsequently precipitated by trichloroacetic acid (TCA). Protein powders were prepared from the TCA-precipitated serum proteins and from the homogenized liver as described earlier (*3*). They were counted on a gas flow counter that had a thin window.

We present our data in terms of relative specific activity (R.S.A.) which is defined as follows (*3*):

$$\text{R.S.A.} = \frac{\text{count/min in 100 mg protein}}{\text{count/min injected per g body wt.}}$$

The values for relative specific activity of the serum albumin fraction and of the liver protein obtained from the four (in some instances three) recipient rats similarly injected and exsanguinated on the same day were averaged and are presented in Fig. 1. A significantly higher relative specific activity is found in the "old" albumins 24 hours after injection, before complete equilibration with the extravascular fluids. The slower rate of equilibration in these animals may be due to their larger size.

After the first day, the decrease of relative specific activity of old and young proteins was almost linear when plotted on semilogarithmic paper. Straight lines (Fig. 1) were calculated from the values between the second and ninth days by means of the method of least squares. Their slope indicates half-lives of 2.9 days for the "young" and 2.8 days for the "old" rat serum proteins of the albumin fraction. The small difference between these two values is not significant in view of considerable individual variations.

The half-lives calculated from the decay rate of the circulating S^{35}-rat serum albumin fraction are apparent half-lives, since they are the result not only of breakdown of the injected material but also of reutilization of breakdown products, S^{35}-amino acids, for the formation of new plasma proteins (*4*). The reutilization of S^{35}-amino acids is clearly indicated by their incorporation into the liver proteins (Fig. 1). However, the true half-life of the serum proteins is only slightly lower than their apparent half-life (*5*).

In evaluating the breakdown of old and young serum proteins, it must be borne in mind that the "young" albumins are formed within 8 hours after injection of S^{35}-amino acids so that their age cannot be more than 8 hours. If neither breakdown nor reutilization of

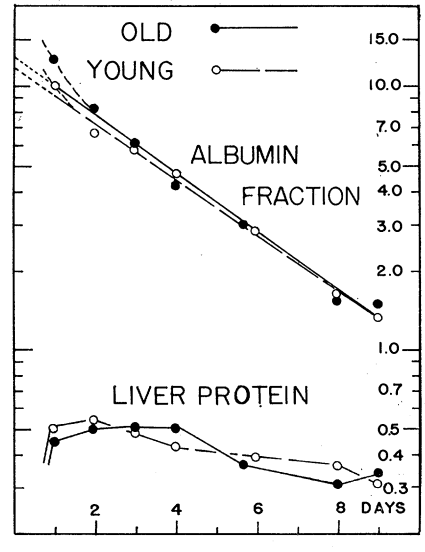

Fig. 1. Relative specific activities of serum albumin fraction and liver protein between the first and ninth days after injection of S^{35}-rat serum protein into rats.

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breakdown products occurred, their average age would be 4 hours. Both processes occur to a small extent, so that the average age is slightly less than 4 hours. The "old" serum proteins are formed within a period of 93.5 hours after injection. Since most of the injected S^{35}-amino acids are incorporated in the first few hours after injection, the average age of the "old" serum proteins at the time of exsanguination is probably between 80 and 90 hours. Although new S^{35}-serum protein is formed later by reutilization of breakdown products, the extent of this process is so small that it can be neglected here.

In spite of the large difference in age between the "young" and "old" proteins, we find no significant difference in their half-lives, nor in their utilization for the formation of liver protein. There is no indication that the age of the molecules has any influence on their rate of breakdown. We conclude, therefore, that the injected serum protein molecules are broken down randomly without any preference for "young" or "old" molecules.

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References and Notes

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Metabolic Reactivation of Rickettsiae by Diphosphopyridine Nucleotide

A previous report from this laboratory (*1*) has shown that *p*-aminobenzoic acid (PABA) forms an adduct with diphosphopyridine nucleotide (DPN^+), rendering DPN^+ unavailable to DPNase. One implication of these experiments has been applied to link two apparently separated phenomena involving rickettsiae. Fifteen years ago (*2*) it was shown that PABA inhibits rickettsial proliferation, and recently Bovarnick (*3, 4*) has shown "reactivation" of "inactivated" typhus rickettsiae by DPN^+. While inconclusive explanations for the PABA inhibition have been offered (*5, 6*), no adequate theory has been presented explaining the "reactivation" role of DPN^+, and certainly neither evidence nor hypothesis has ever been proposed relating these widely separated observations. In this preliminary report (*7*) evidence is presented that the PABA-DPN^+ adduct (*1*) prevents rickettsial dehydrogenase activity and that such activity is restored when exogenous DPN^+ is added to rickettsiae.

Table 1. Effect of PABA on the reduction of DPN^+ by rickettsial extracts. PABA, 8 μmole/ml; 3,5-dimethyl-4-aminobenzoic acid, 8 μmole/ml; exogenous DPN^+, 8 μmole/ml; L-K-malate, 0.3*M*; buffer, tris, 0.05*M*, *p*H 7.4; *C. burnetii* extract, 1.2 μmole of DPN^+ per milliliter; *R. prowazeki* extract, 1.86 μmole/ml; volume made to 3 ml with distilled water; temperature, 35°C. System I was incubated for 16 hr, then system II was added; this mixture was incubated for 2 hr, and then system III added; the final mixture was incubated 45 min; ΔOD was measured at 340 mμ in Beckman B spectrophotometer, and represents final value.

System		1 (ml)	2 (ml)	3 (ml)	4 (ml)	5 (ml)	6 (ml)
I	Enzyme	0.3	0.3	0.3	0.3	0.3	0.3
	Buffer	0.5	0.5	0.5	0.5	0.5	0.5
	PABA			0.1			0.1
	3,5-diMe-PABA				0.1		
II	Exogenous DPN^+					0.2	0.2
III	Malate		0.5	0.5	0.5	0.5	0.5
ΔOD							
	C. burnetii	0	0.19	0.04	0.19	0.35	0.14
	R. prowazeki	0	0.28	0.03	0.28	0.43	0.23

Purified suspensions of *Coxiella burnetii* (*8*), LD_{50} of 10^{-3}, and the Madrid E strain of *Rickettsia prowazeki*, LD_{50} of 10^{-8} to 10^{-9} (*7*), were separately treated in the Raytheon 9 KC sonic oscillator, and enzyme extracts were prepared. The DPN^+ contents of the rickettsial extracts were determined according to the procedure described by Racker (*9*). The cell-free preparation from *Coxiella burnetii* contained 0.36 μmole of DPN^+ per 0.3 ml, and the *Rickettsia prowazeki* had 0.56 μmole of DPN^+ per 0.3 ml. These amounts were equivalent under the conditions employed to ΔOD's at 340 mμ of 0.17 and 0.205, respectively. The enzymes were treated with PABA, and then reacted with malate, as is shown in Table 1.

The data unequivocally show that, after reaction with PABA, rickettsial DPN^+ is not reduced and that such inhibition is partially relieved by the addition of exogenous DPN^+ to the system. The addition of 0.8 μmole of PABA to *Coxiella burnetii* produced 79 percent inhibition. When 1.6 μmole of exogenous DPN^+ was added, the inhibition was reduced to 60 percent. For Madrid E, under identical conditions, 89 percent PABA inhibition obtained; the inhibition fell to 46 percent upon addition of DPN^+. When 0.8 μmole of 3,5-dimethyl-4-aminobenzoic acid was added instead of PABA, no inhibition of dehydrogenase activity was observed. This is in agreement with theory, since the methyl groups on the 3- and 5-positions block the formation of a negatively charged structure at these sites (*1*). These experiments have now been repeated three times, with similar results.

Previous work in these laboratories (*1*) has shown that DPN^+ chemically interacts with PABA, forming an adduct. Due to resonance, the unshared pair of electrons of the amino group of the PABA molecule can be shared by the *ortho* and *para* carbon atoms of the ring, and therefore the molecule becomes susceptible to attack by an electrophilic agent such as DPN^+. The negatively charged *ortho* carbon of the PABA adds to the positively charged *para* carbon of the pyridinium moiety of DPN^+. The evidence presented in this report suggests an explanation for the inhibition of rickettsiae by PABA, based on the above reaction. By forming an adduct with rickettsial DPN^+, PABA effectively inhibits cellular reactions involving this vital coenzyme, thus inhibiting rickettsial metabolism. The addition of exogenous DPN^+ to such a system relieves inhibition. Other explanations have been previously offered for the rickettsiostatic effect of PABA. Greiff and Pinkerton (*5*) employed PABA on rickettsial-infected embryonated eggs. They proposed that PABA participated in undescribed respiratory mechanisms. In effect, the PABA acted by enhancement of host cell respiration, with resulting deleterious effect on rickettsiae. Snyder and Davis (*6*) demonstrated that the addition of *p*-hydroxybenzoic acid to rickettsiae-infected embryonated eggs relieved PABA inhibition of rickettsiae. They claimed that *p*-hydroxybenzoic acid competitively reverses the action of PABA on rickettsiae. In the work reported in this paper, the inhibitory effect of PABA has been examined in enzyme extracts of purified, nonproliferating rickettsiae. Under such