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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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

conditions the complicating reactions of the host cells are removed.

A report is now in preparation describing analogous experiments in our laboratories, in which sulfanilamide was employed instead of PABA. Sulfanilamide is similar to PABA not only in structure but in that similar resonant structures exist in which the positions *ortho* to the amino group possess a formal negative charge. Sulfanilamide inhibitions similar to our already described PABA inhibition have been observed with Gram-positive and Gram-negative bacteria as well as with cell-free preparations of rickettsiae. As in the case of PABA, these inhibitions are relieved by the addition of DPN+. Andrewes *et al.* (*10*) found that *p*-sulphonamidobenzamidine and *p*-sulphonamidobenzamidoxime were potent antirickettsial agents. It is of interest to note that both of these compounds can give rise to resonant structures bearing formal negative charges in the *ortho* positions, which should permit addition to the *para* position of DPN+. Among other things, Andrewes *et al.* (*10*) proposed that nuclear-substituted sulfonamides have "the specific power of adding on to some unknown structure in the enzyme and thus affecting its activity." They further state "it is significant that a free amino group is essential in all drugs derived from sulphanilamide for activity." The latter statement can now be reexamined in light of reference (*1*). Cell permeability has been found in our laboratories to play a significant role in the antibacterial property of sulfanilamide against rickettsial infections.

The evidence presented suggests a simultaneous explanation for both the inhibition of rickettsiae by PABA and the DPN+ reactivation phenomenon of Bovarnick (*4*). Both the Snyder and Bovarnick phenomena seem to have DPN+ involvement as a common factor, the PABA inhibition phenomenon being due to inactivation of rickettsial DPN+ while Bovarnick's reactivation phenomenon restores the DPN+ to rickettsiae in which DPN+ has been eliminated by physical methods. The implications of the data presented, insofar as they offer an additional mode of sulfonamide action, are apparent and emphasize the biological significance of the chemical reactivity of DPN+.

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Color Reaction of Bilirubin with Sulfuric Acid: a Direct Diazo-Reacting Bilirubin Sulfate

A color reaction of bilirubin with the Liebermann-Burchard reagent was first observed in 1936, but was not reported or studied in detail at that time. It was noted then, however, that following this reaction the bilirubin became soluble in water. Unfortunately the significance of this change in relation to the diazo reaction was not appreciated at that time.

This reaction has now been studied in some detail and has been found to be of special interest because it yields an anionic complex of bilirubin which exhibits a prompt, direct diazo reaction. Except for a brief reference in a recent paper of mine (*1*), no mention of the color reaction has been found in the literature. The reaction occurs more slowly with bilirubin and sulfuric acid alone, and it is evident that the acetic anhydride in the Liebermann-Burchard reagent serves only as a vehicle to bring the sulfuric acid into rapid contact with the bilirubin. The addition of 1 ml of the acetic anhydride-sulfuric acid (10:1) reagent to 5 ml of a chloroform solution of bilirubin is followed promptly by a dark red color, the reaction mixture then exhibiting a strong absorption band at maximum 540 mμ. If the solution is shaken in a separatory funnel with distilled water, the red color at once changes to brown-yellow, and it is evident that much of the pigment has now become water soluble, this fraction entering the aqueous phase.

The remainder of the bilirubin in the chloroform phase may be converted to the water-soluble type by successive addition of small amounts of the Liebermann-Burchard reagent and shaking with water. It is evident that in the course of the reaction small fractions of the bilirubin are converted to a dark brown pigment, probably bilifuscin, which precipitates at the interphase. Biliverdin is also formed in considerable amount, and it continues to form, so that the aqueous, initially yellow or brownish yellow soon becomes greenish brown.

After separation of the pooled aqueous fraction from any entrained chloroform, by filtration, the van den Bergh reaction is prompt and direct in type. Unlike the behavior of starting bilirubin, its behavior is polar, as is shown by reverse phase chromatography on siliconized *kieselguhr*, by the method of Cole and co-workers (*2*). The substance corresponds in chromatographic behavior to their pigment II. The polar pigment is readily separated from biliverdin and other impurities by adsorption on a column of aluminum oxide. The diazo-reacting pigment is held at the top of the column, the impurities coming off with the effluent. The pigment is then readily eluated with 0.1*N* sodium hydroxide solution. It retains its polar character and is not extracted by chloroform upon acidification of the eluate.

Thus far it has been impossible to crystallize this prompt, direct diazo-reacting compound. It is quite labile, readily undergoing oxidation to biliverdin and bilifuscin. Due to its polar behavior and water solubility, it is difficult to transfer to organic solvents, although it is extracted in part by *n*-butanol at *p*H 4 to 5.0. The polar, prompt and direct-reacting pigment is readily diazotized in the initial aqueous solution after the *p*H is adjusted to 4.0. The azo compound is extracted by *n*-butanol and may then be compared with the azo dipyrryl compounds of free bilirubin and of the conjugated bilirubin of human bile, which, according to recent evidence, is mainly an ester diglucuronide (*3–6*). The crude bilirubin glucuronide used in the present study was prepared by Malcolm Campbell (*7*). For the present comparison, Schmid's paper chromatographic method (*3*) was used. The concentrated butanol solutions of the three azo compounds were run on the same strip (Whatman 3 MM), ascending chromatography being achieved with *n*-propionic acid, methyl ethyl ketone, and water (25:75:30). With a solvent front of about 30 cm, the R_f for the azo pigment from crystalline bilirubin is 0.55, that of the glucuronide 0.40. The bilirubin sulfate regularly exhibits two zones, 0.30 (red) and 0.22 (purple). The basis for this has not been determined and is receiving further study.

It has been possible to show that the azo pyrryl compound, after paper chromatography as described, contains sulfate, while that of the glucuronide, under the same conditions, does not. For this demonstration (*8*) a considerable amount of each azo pigment was chromatographed, then eluted from the paper by