there should be 25 μM of sites available. This is more than adequate to account for the 3–5 μM of specific binding observed.

The formulation of a general theory of amino acid incorporation into proteins on the basis of these observations with proline and methionine would not be justified. However, any model of protein synthesis must provide a means of selectively locating amino acids and must provide the energy to form the peptide bonds. These studies of proline and methionine incorporation give an experimental demonstration of the operation of these two important processes in the synthesis of protein.

* Note added in proof: A report of similar studies carried out nearly simultaneously by G. N. Cohen and H. V. Rickenburg appears in *Comptes rendus des seances de l'Academie des sciences*, **240**, 2086.

[1] Various technical details such as culture media, chromatographic solvents, special chromatographic methods, and procedures for chemical fractionation of the cells are described in Roberts, Abelson, Cowie, Bolton, and Britten, "Studies of Biosynthesis in *Escherichia coli*," *Carnegie Inst. Washington Publ.*, No. 607, 1955.

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*VIRULENT-AVIRULENT CONVERSIONS OF RICKETTSIA RICKETTSII IN VITRO**

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Earlier reports[1, 2] from this laboratory have demonstrated that virulent strains of *Rickettsia rickettsii*, the etiological agent of Rocky Mountain spotted fever (RMSF), exist under certain conditions in an avirulent phase in its arthropod vector, *Dermacentor andersoni*. This phase is virulent for chick embryos but avirulent for guinea pigs and many other laboratory animals.[2] Previous experiments also showed that temperature[1, 3] and the molting process[2] of the arthropod were important in controlling the virulence of the rickettsiae in the arthropod vector. The correlation between the virulent-to-avirulent changes and the epidemiological behavior of *R. rickettsii* has been discussed previously.[2]

In this paper is reported (*a*) the in vitro conversion of virulent *R. rickettsii* to an avirulent state by para-aminobenzoic acid (PABA); (*b*) the in vitro reactivation or conversion of the PABA-avirulent and arthropod-avirulent rickettsiae[1] to the virulent form by the addition of Coenzyme I (CoI) or Coenzyme A (CoA); and (*c*) the observation that the state of virulence for guinea pigs parallels the adsorptivity of *R. rickettsii* to minced guinea pig tunica.

RESULTS

In Vitro Conversion of Virulent R. rickettsii to an Avirulent State by PABA.— In Table 1 are given the results of a typical experiment in which a 10 per cent virulent yolk sac suspension of *R. rickettsii*[1] in sucrose-phosphate-glutamate (SPG) solution[4] was incubated at 25° C. for 60 hours with 5 mg/ml of PABA. Although

in the absence of PABA the egg infectivity of the agent was lost by the incubation, in the presence of PABA 4 of the 6 logs of egg infectivity survived but the material failed to produce RMSF in guinea pigs.[5] Para-hydroxybenzoic acid (POB), CoI, and CoA mixed with PABA prevented this loss of virulence. POB is known[6] to reverse the rickettsiostatic effect of PABA. CoII could not replace CoI under the conditions shown in Table 1.

TABLE 1

EFFECT OF POB, CoI, AND CoA, ON PABA-TREATED RICKETTSIAE

Sample*	Addition (Mg/ml)	Titer after 60-hour Incubation (Egg LD_{50})	Spotted Fever in Guinea Pigs*	Adsorption to Guinea Pig Tunica (Egg LD_{50})†
1. Virulent phase	None	$<10^{0.5}$	0/6	. . .
2. Virulent phase	5.0 PABA	$10^{4.3}$	0/6	$<10^{0.5}$
3. Virulent phase	5.0 PABA + 2.0 POB	$10^{4.5}$	6/6	$10^{2.3}$
4. Virulent phase	2.0 POB	$10^{3.6}$	6/6	$10^{2.3}$
5. Virulent phase	5.0 PABA + 0.5 CoI	$10^{4.6}$	6/6	$10^{2.5}$
6. Virulent phase	5.0 PABA + 0.3 CoA	$10^{4.2}$	6/6	$10^{2.3}$

* Numerator shows number of guinea pig developing spotted fever. All guinea pigs except those of sample 1 received approximately $10^{3.0}$ egg LD_{50} intraperitoneally (see W. H. Price, *Am. J. Hyg.*, **58**, 248–268, 1953). All samples were incubated 60 hours at room temperature before being inoculated into the animals. The initial titer was $10^{6.5}$ egg LD_{50} before incubation.

† Guinea pig tunica was minced into approximately 1.0-mm. pieces and washed 4 times with Hanks' solution. Samples weighing 0.5 gm. were put into 25-ml. Erlenmeyer flasks, and 2.0 ml. of inoculum containing the appropriate rickettsial suspensions after the 6-hour incubation period were pipetted into each flask and shaken gently at room temperature for 90 minutes. The titer of each inoculum was approximately $10^{4.0}$ egg LD_{50}. After a 90-minute adsorption period the tissues in the various flasks were washed 4 times with 10 ml. of SPG solution. No rickettsiae were detected in the final wash fluid as determined by egg titration (*ibid.*). The various samples were then ground in a mortar in a total volume of 5 ml. of SPG solution, centrifuged at 800 × *g* for 5 minutes, and the supernatant fluid then titered in chick embryos (*ibid.*). The addition of CoI to the avirulent phase results in as many rickettsiae being adsorbed to guinea pig tunica as are found when fully virulent rickettsiae are studied. When 100 egg LD_{50} of the virulent phase was injected intraperitoneally along with 5 mg. of PABA, the guinea pigs developed typical spotted fever. As a further control, 10 per cent normal yolk sac was incubated with 5 mg. of PABA for 60 hours. At this time 10 egg LD_{50} of the virulent phase was added to the suspension, mixed well, and injected intraperitoneally. The guinea pigs developed typical spotted fever. It is clear that the avirulence of sample 2 cannot be due to PABA being carried over in the inoculating suspension.

After reactivation had taken place, the suspensions could be diluted 1,000-fold and still cause RMSF when injected into guinea pigs. Since these conversions in virulence take place in vitro and occur under conditions which are unfavorable for rickettsial multiplication, it would appear that more than 99 per cent of the viable organisms are concerned in these transformations. If as little as 3 egg LD_{50} of the virulent rickettsiae are mixed with 100 egg LD_{50} of the PABA-treated avirulent suspensions, the injection of such suspensions into guinea pigs results in RMSF.

In Vitro Reactivation or Conversion of Tick Avirulent to Virulent R. rickettsii.— Table 2 illustrates the results of experiments in which tick avirulent rickettsiae[1] were incubated 6 hours at 25° C. with CoI or CoA. With either coenzyme the rickettsial agent took on the properties of the virulent phase for guinea pigs. Incubation with POB did not produce virulent rickettsiae. Experiments not shown here, with various preparations of CoI, showed that their capacity to convert the avirulent to virulent rickettsiae paralleled the CoI activity.

In addition, destruction of the CoI with snake venom pyrophosphatase[7] also destroyed the material which changed the avirulent into the virulent form. All this is strong evidence that CoI[8] is the active component in the in vitro conversion mechanism. This is of interest in view of the stimulatory effect of this coenzyme on the respiratory of *R. rickettsii*.[8] Bovarnick and Allen[9] reported that CoI and CoA increase the infectivity of the E strain of *R. prowazeki* for both eggs and animals

under conditions of freezing and thawing which had destroyed its infectivity. This situation is different from the reactivation of *R. rickettsii* in that the avirulent phase of *R. rickettsii* is virulent for chick embryos.[1]

TABLE 2

EFFECT OF CoI, CoA, AND POB ON THE VIRULENCE OF THE TICK AVIRULENT PHASE

Sample	Additions (Mg/ml)	Titer after 6-Hour Incubation (Egg LD_{50})	Spotted Fever in Guinea Pigs*	Adsorption to Guinea Pig Tunica (Egg LD_{50})†
1. Avirulent phase	None	$10^{3.5}$	0/6	$<10^{0.5}$
2. Avirulent phase	0.500 CoI	$10^{4.3}$	6/6	$10^{2.3}$
3. Avirulent phase	0.300 CoA	$10^{3.7}$	6/6	$10^{1.6}$
4. Avirulent phase	2.0 POB	$10^{4.6}$	0/6	$<10^{0.5}$
5. Virulent phase	None	$10^{3.5}$	6/6	$10^{2.1}$
6. Virulent phase	2.0 POB	$10^{4.5}$	6/6	$10^{2.4}$

* Numerator shows number of guinea pigs injected intraperitoneally with $10^{3.5}$ egg LD_{50} of the various samples which developed spotted fever. Samples 1–6 were incubated 6 hours at 30° C. before being injected into the animals. Similar results were obtained if the suspensions were diluted 100-fold and injected into guinea pigs. The initial titer before incubation was $10^{5.6}$ egg LD_{50} in all samples, 50 ticks being ground in 50.0 ml. of SPG solution. The incubation period is necessary for the conversion of the avirulent tick suspension to the virulent phase. If CoI is added to the avirulent tick preparation and this suspension immediately inoculated intraperitoneally into guinea pigs, the rickettsiae remain avirulent and the guinea pigs develop no clinical symptons of RMSF.

†The adsorption experiments were carried out as described in Table 1.

In Vitro Conversion with Normal Tick Extracts.—When normal (uninfected) adult ticks are incubated at 35° C. for 24 hours and then ground up, the suspension will raise the virulence of *R. rickettsii* under the conditions described in Table 2. This same is true for normal nymphs that have received a blood meal. No such activity can be found in ticks kept at low temperatures. Thus, from those two phases of *D. andersoni* in which reactivation takes place naturally, it is possible to prepare extracts which will duplicate the reactivation in vitro. Since the various phases of the tick life-cycle are under the control of one or more molting hormones, it is suggested that this probably plays a direct or indirect role in the formation of the active metabolites which convert the rickettsiae from one form to another, since it has been shown that the virulence of *R. rickettsii* is decreased when a nymph of *D. andersoni* infected with a virulent strain of *R. rickettsii* molts to the adult stage,[2] and this low virulent phase can be raised to a more virulent phase by the addition of tick extracts as described above.

Relationship of Virulence to Adsorption.—Since the virulent strains of *R. rickettsii* grow very well in the tunica of guinea pigs, a suspension of minced guinea pig tunica was used to test the adsorption of the various rickettsial preparations. The results are shown in Tables 1 and 2. It may be seen that the avirulent phase showed very low adsorption to the minced tunica and that whenever the virulence increased, as shown by the production of disease in guinea pigs, the adsorption to tunica suspensions increased. This suggests that the avirulence is due to failure to adsorb, and apparently this requires CoI or CoA or perhaps other biological materials.

CONCLUSION

The above results suggest, first, that the changes in virulence of *R. rickettsii* can be produced in vitro and need not be due to selection, mutation (in the usual sense of the word), or a combination of these two factors, since they take place under conditions which are considered unfavorable for rickettsial multiplication. Further-

more, the results show that more than 99 per cent of the organisms are involved in these virulence changes. Second, the data also indicate that the organic metabolic compounds, CoI, CoA, PABA, and POB, affect the attachment of *R. rickettsii* to guinea pig tunica, and, if the rickettsiae cannot attach, they obviously cannot infect a host cell. Such information might prove useful in a search for chemotherapeutic agents against rickettsiae and large viruses. Third, the results show that the virulence of an arthropod-borne microparasite can be modified by specific metabolic substance(s) formed by the arthropod and that the formation of this substance(s) may be under the control of a hormone of the arthropod.

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[1] W. H. Price, *Science*, **118**, 49–52, 1953.

[2] W. H. Price, in *Dynamics of Virus and Rickettsial Infections*, ed., Hartman, Horsfall, and Kidd (New York: Blakiston Co., 1954), pp. 164–183.

[3] R. R. Spencer and R. R. Parker, *Hyg. Lab. Bull.*, **154**, 1–116, 1930.

[4] M. R. Bovarnick, J. C. Miller, and J. C. Snyder, *J. Bacteriol.*, **59**, 509–522, 1950.

[5] One egg LD_{50} of the virulent phase is approximately equal to 1 guinea pig ID_{50} as determined by clinical symptoms (see W. H. Price, *Am. J. Hyg.*, **58**, 248–268, 1953).

[6] J. C. Snyder and B. D. Davis, *Federation Proc.*, **10**, 419 (1951.

[7] Pyrophosphatase was kindly supplied by Dr. G. Rafter. The CoI in most experiments was 95 per cent pure and was obtained from the Nutritional Biochemicals Corporation, Cleveland, Ohio. The CoA was obtained from the same firm and was 75 per cent pure.

[8] W. H. Price, *Am. J. Hyg.*, **58**, 248–268, 1953.

[9] M. R. Bovarnick and E. G. Allen, *J. Gen. Physiol.*, **38**, 169–179, 1954.