at about 0.02 percent is consistent with what would be expected if the genetic code were degenerate. Despite the complicated operations required to obtain this number it is remarkably reproducible. In independent experiments values of 0.019 percent and 0.023 percent were obtained in this laboratory. In addition, Goodman and Rich (*13*) in a very similar experiment found a value of 0.024 percent. Therefore, the value can be given some credence. If anything, it is probably an underestimation since conditions for hybridization may not be optimal and the treatment with ribonuclease may slowly remove some s-RNA that had entered into the complex.

The expected saturation level can be estimated from the molecular weight equivalent of the genome of *Escherichia coli* (4×10^9) and the number of different kinds of s-RNA molecules, each of which has a molecular weight of 2.5×10^4. If the genetic code is not degenerate, each amino acid is coded by only one triplet which implies that there are only 20 different s-RNA molecules. If the code is degenerate, more than 20 will be needed in the dictionary. The plateau predicted by the non-degenerate case is 0.01 percent. The fact that it is at least twice as high suggests that some amino acids are identified with more than one s-RNA molecule. This possibility is consistent with the accumulating evidence for degeneracy which has emerged from triplet identifications (*14, 15*) and agrees with the multiplicity of types of s-RNA for individual amino acids (*16, 17*). That this multiplicity is the physical basis for degeneracy has been demonstrated by Weisblum *et al.* (*18*).

The data available (*19, 20*) suggest that the genetic dictionary is universal, or nearly so. However, the coding triplets probably occupy only a small proportion of the s-RNA strands. Although the function of the non-coding stretches of approximately 70 nucleotides is as yet unknown. They provide the opportunity for biological individuality by sequence variation without disturbing the functioning of the universal language. The specificity of complex formation in the present experiments shows that this opportunity was not neglected in the course of biologic evolution. Thus, although the s-RNA of *E. coli* can translate the genetic message of a rabbit into hemoglobin (*19*), the s-RNA can be uniquely identified with the genome of its origin.

Ribosomal RNA appears to have the same combination of genetic uniqueness and use unrestricted by specificity requirements. Ribosomes are comparatively indifferent (*14–16*) to the origin of the genetic messages to which they respond. However, their sequences are unique, since they hybridize readily only to homologous DNA (*3*).

One other feature is shared by these two molecular species. Although their sequences vary, the over-all base composition is remarkably similar in a variety of organisms. Thus, ribosomal RNA from *Pseudomonas aeruginosa* with a DNA containing 64 percent guanine-cytosine is indistinguishable from that of *Bacillus megaterium*, the DNA of which contains 44 percent guanine-cytosine. We are faced with the paradox that two sets of cistrons, those for s-RNA and ribosomal, have resisted the drift toward different average base compositions (*21*).

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References and Notes

1. M. W. Nirenberg, J. H. Matthaei and O. W. Jones, *Proc. Natl. Acad. Sci. U.S.* **48**, 104 (1962); F. Chapeville, F. Lipmann, G. von Ehrenstein, B. Weisblum, W. J. Ray, Jr., S. Benzer, *ibid.* **48**, 1086 (1962).
2. B. D. Hall and S. Spiegelman, *ibid.* **47** 137 (1961).
3. S. A. Yankofsky and S. Spiegelman, *ibid.*, **48**, 1069 (1962); *ibid.*, 48, 1466 (1962).
4. R. H. Doi and S. Spiegelman, *Science*, **138**, 1270 (1962).
5. A. Gierer and G. Schramm, *Nature*, **177**, 702 (1956).
6. J. D. Mandell and A. A. Hershey, *Anal. Biochem.* **1**, 66 (1960).
7. M. Hayashi and S. Spiegelman, *Proc. Natl. Acad. Sci. U.S.* **47**, 1564 (1961).
8. J. Spencer, W. Fuller, M. H. F. Wilkins, G. L. Brown, *Nature* **194**, 1014 (1962).
9. D. S. McCully and G. L. Cantoni, *J. Mol. Biol.* **5**, 80 (1962).
10. J. Hurwitz, J. J. Furth, M. Malamy, M. Alexander, *Proc. Natl. Acad. Sci. U.S.* **48**, 1222 (1962).
11. E. Reich, R. M. Franklin, A. J. Shatkin, E. L. Tatum, *ibid.* **48**, 1238 (1962).
12. E. Fleissner and E. Borek, *ibid.* **48**, 1199 (1962).
13. H. M. Goodman and A. Rich, personal communication.
14. R. G. Martin, J. H. Matthaei, O. W. Jones, M. W. Nirenberg, *Biochem. Biophys. Res. Commun.* **6**, 410 (1962).
15. J. F. Speyer, P. Lengyel, C. Basilio, S. Ochoa, *Proc. Natl. Acad. Sci. U.S.* **48**, 441 (1962).
16. P. Berg and V. Lagerkvist, *Acides Ribonucleiques et Polyphosphates*, (C.N.R.S., Paris 1962).
17. B. P. Doctor, J. Agper, R. W. Holley, *J. Biol. Chem.* **236**, 117 (1961); N. Sueoka and T. Yamane, *Proc. Natl. Acad. Sci. U.S.* **48**, 1454 (1962).
18. B. Weisblum, S. Benzer, R. H. Holley, *ibid.* **48**, 1449 (1962).
19. G. von Ehrenstein and F. Lipman, *ibid.* **47**, 941 (1961).
20. A. Tsugita, H. Fraenkel-Conrat, M. W. Nirenberg, J. H. Matthaei, *ibid.* **48**, 846 (1962); B. Weinstein and A. N. Schechter, *ibid.* **48**, 1686 (1962); E. S. Maxwell, *ibid.* **48**, 1639 (1962).
21. Aided by grants from the U.S. Public Health Service, National Science Foundation, and the Office of Naval Research. One of us (D.G.) is an Upjohn predoctoral fellow in molecular genetics.

16 November 1962

Anthrax Toxin: Causative Agent in the Death of Rhesus Monkeys

Abstract. Anthrax toxin was present in the blood obtained from rhesus monkeys at the terminal stage of anthrax and was also present in increasing amounts in the lymph as death approached. When sterile toxin produced in vitro was injected in sufficient amounts, monkeys died unless they were injected with specific antiserum. Death with symptoms of toxemia occurred within 2 hours after injection of 10^{11} spores, whereas 10^{10} spores or less required 20 or more hours of growth in vivo to cause death.

In 1955 Smith *et al.* were able to demonstrate toxin in the blood of guinea pigs dying from anthrax (*1*). Previous investigators (before Smith *et al.*) had attempted this proof, but failed. Except for Middleton and Standen (*2*), who found toxin in the blood of two of four monkeys just before they died of anthrax, no one has determined whether toxin is always associated with terminal anthrax or whether specific antiserum neutralizes toxin and could be useful in treatment for the disease. It is not known whether whole toxin is a better antigen than "protective antigen," one of its components (*3, 4*), or whether a toxoid can be prepared that could be used for immunization. In this paper we report initial experiments which suggest that rhesus monkeys infected with anthrax die of toxemia. This inference is supported by four observations: (i) toxin is present in the blood of monkeys at the terminal stage of anthrax infection; (ii) toxin increases in concentration as the disease progresses in both the lymph and blood and reaches a maximum at death; (iii) sterile toxin alone causes death of the monkey; and (iv) a rapid toxemic death occurs after an injection of 10^{11} *Bacillus anthracis* spores.

Blood and lymph were obtained by the procedures of Hodges and Rhian (*5*). Lymph was collected continuously and blood was drawn as desired. Changes in the concentration of toxin in the lymph and blood after challenge was determined by intravenous injection of Fischer-344 rats by the method of Beall *et al.* (*4*). Bacilli in these fluids were counted by the dilution plate method or by direct quantitative observation, as described by Keppie *et al.* (*6*). The crude in vitro toxin and the antiserum used were prepared by the method of Beall *et al.* (*4*).

While we were doing other work on pathogenesis, anthrax toxin was found in the blood of 12 out of 15 rhesus monkeys in the terminal stages of an-

Table 1. Bacilli and toxin in lymph from monkeys infected with *B. anthracis*. The number of bacilli is the number in 1 ml of lymph. Presence of toxin is signified by the number of minutes required for rats to die after injection of lymph from the infected monkeys.

Interval before death of monkeys (hr)	No. of bacilli (10^6/ml of lymph)		Interval (min) until death of rats injected with lymph from	
	Monkey 1	Monkey 2	Monkey 1*	Monkey 2†
17	0.2		*S*‡	
15		41		
11	71	12	360	*S*
9	69	24	233	*S*
8		15		*S*
7	168	18	143	240
5	85	19	126	237
3	88	15	144	180
Death	69	26	76	177

* Mean time to death of two rats. † Time to death of one rat. ‡ Rat survived.

thrax. We have now observed a relationship between the final concentration of bacilli in the blood, the time until death after challenge, and the presence of toxin. When death occurred early after challenge, the final concentration of bacilli was high and toxin was present; the converse was true when the interval before death was longer. These observations probably account for absence of toxin in those three monkeys in which toxin was not found.

The concentration of toxin in the lymph of two monkeys rose to a maximum at death, as shown in Table 1. The thoracic lymph duct and the jugular vein of the first monkey were cannulated, and 10^5 virulent spores were injected intradermally. The site of the inoculation was drained by the popliteal lymph node. Lymph was collected regularly until the animal died (41.5 hours). Bacilli were first observed in the lymph 24.5 hours after challenge, or 17 hours before death. At this time the test for toxin was negative. Presence of toxin in the lymph sample taken at the 11th hour before the death of the monkey was demonstrated by the death of an injected test rat. The time after injection required for toxin to cause death in rats decreased regularly in each successive sample ($p = .001$). The final sample caused death in 76 minutes, or one-fifth the time interval that followed injection of the initial toxic sample. Bacilli were not observed in the blood until 11.5 hours before death. The final blood sample was not toxic for rats.

In the second monkey the right lymph duct, which drains a major portion of the lungs, was cannulated and the monkey was challenged by a dose of 3×10^5 spores contained in an aerosol. The monkey died 57 hours later. Bacilli in the lymph, in a low concentration, were first present 10 hours after challenge, and intermittently for 38 hours, at which time the concentration increased rapidly to 4×10^7 cells per milliliter of lymph. The count remained nearly constant at about 2×10^7 bacilli per milliliter. The lymph sample obtained 8 hours before death was not toxic but a sample collected 1 hour later was toxic. The decrease in the response time of the rats, about 240 minutes to 180 minutes for successive lymph samples, was statistically significant ($p = .10$). Although the level of significance is low, the regression does indicate that there was an increase in toxin concentration in this body fluid during the period of observation. Blood taken at the terminal stage also was toxic for rats. These rats were challenged with only 0.5 ml of lymph whereas 1.0 ml of lymph was injected into each rat from the first monkey. Therefore these two lymph assays were not directly comparable.

The apparent differences in the toxicity of the final blood samples were expected. We believe that toxin was present in the blood of the intradermally challenged monkey but that it was below a demonstrable level. Toxin was demonstrated in the blood of the monkey challenged with the aerosol mixture even though the lymph drained to the exterior. This may indicate that there was another route of entry of toxin into the blood. Although toxin in the blood of the cannulated monkeys could have arrived by several pathways, the most logical is the establishment of pathological secondary "lympho-venous" communications in the lymph nodes. Such a mechanism was suggested by Malek *et al.* from their studies of similar conditions in sheep (*7*). If the phenomenon reported by Malek *et al.* occurs in monkeys, the amount of toxin entering the blood directly would be proportional to the number of lymph nodes draining a particular site of challenge. The number of lymph nodes draining the lungs of a monkey was far greater than the number of lymph nodes which drained the site of inoculation, below the popliteal node, in the intradermally challenged monkey. The number of lymph nodes and the proportional development of such secondary pathological lympho-venous communications between the two lymph systems and the blood may account for the absence of toxin and low concentration of organisms in the terminal blood of the intradermally challenged monkey.

The data show that toxicity is proportional to the concentration of organisms in the body fluids. The lymph of both monkeys showed parallel increases of organisms and toxin. The blood of the first showed a negligible increase of organisms and no toxicity, whereas the blood of the second showed a substantial increase in organisms accompanied by demonstrable toxicity. This is the first demonstration for such an accumulation of toxin in body fluids of monkeys and complements the results obtained on toxin in the final samples of blood from guinea pigs dying of anthrax (*1*).

To show that anthrax toxin alone was sufficient to cause the death of these animals, sterile toxin was produced in vitro (*8*); its potency was such that 1 ml killed rats in about 80 minutes. The toxin (250 ml) was injected intracardially into a 16-pound rhesus monkey with no immediately apparent ill effects. However, 21 hours later, the monkey died with partial paralysis of upper extremities, extreme respiratory distress, and massive pulmonary edema. The two latter signs are often associated with toxic deaths in rats.

In another trial, three 8-pound rhesus monkeys were challenged intravenously with 200, 250, and 400 ml, respectively, of sterile anthrax toxin that had been produced in vitro. A fourth monkey which received 250 ml of toxin was treated with three successive 50-ml injections of antiserum, one given immediately, and the others ½ and 21 hours after toxin injection. The animal which received 200 ml survived; the animals challenged with 250 and 400 ml died in 60 and 30 hours, respectively. The animal that received anthrax antiserum survived. These data indicate a dose-reponse relationship between anthrax toxin and death of the rhesus monkey. They also show that toxin is neutralized by antiserum. The volume of liquid administered, while large, did not noticeably affect the monkey, since the animal which received the antiserum, and survived, received just as much liquid with the toxin as the monkey which died and, in addition, received 150 ml of antiserum.

Additional evidence that death in

the monkeys had been caused by toxemia was provided by a dose-response curve for monkeys challenged intravenously with 10^5 to 10^{11} spores. The dose-response curve for doses from 10^5 to 5×10^{10} spores shows that death occurred in progressively shorter times from 50 to 20 hours respectively. Three monkeys injected with 10^{11} spores, however, died within 2 hours with symptoms of toxemia. On the assumption that each multiplication of a bacillus produces a unit of toxin, it follows that 10^{11} germinating spores would produce 10^{11} units of toxin, a quantity that was rapidly lethal for monkeys. Since 10^{11} particles could be toxic themselves, the same inoculum of 10^{11} spores was reduced in viability to the equivalent of 10^{10} spores by heat. On injection of this inoculum, rapid death did not result, and the course of the disease corresponded to that for 10^{10} viable spores. We conclude that rapid death was caused by an amount of toxin suddenly released by the germination of 10^{11} spores, but the germination of 5×10^{10} or fewer spores released insufficient toxin to be lethal immediately and the disease progressed by a different course.

By four different methods we have shown that toxin is important in causing death of monkeys infected with *B. anthracis* or challenged with crude toxin. The importance of toxin in affecting the pathogenic course, and therefore the treatment of anthrax in man, is yet to be determined. It seems probable that the effect of toxin in man is the same as the effect in other animals. Since the introduction of antibiotics, treatment has been antibacterial. Our experiments suggest that treatment also be directed toward overcoming the effects of toxin or toward preventing its formation. Virtually all Russian recommendations on treatment of any type of anthrax infection put primary emphasis on use of antiserum (*9*). Since "respiratory" anthrax, if identified at all, is typically not identified until septicemia is well advanced, the development and use of specific antitoxin seems warranted. Similar conclusions were drawn by Plotkin *et al.* from their observations on the New Hampshire epidemic of inhalation anthrax (*10*).

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References and Notes

1. H. Smith, J. Keppie, J. T. Stanley, *Brit. J. Exptl. Pathol.* **36**, 460 (1955).
2. G. K. Middleton and A. C. Standen, *J. Infect. Diseases* **108**, 85 (1961).
3. J. T. Stanley and H. Smith, *J. Gen. Microbiol.* **26**, 49 (1961).
4. F. A. Beall, M. J. Taylor, C. B. Thorne, *J. Bacteriol.* **83**, 1274 (1962).
5. D. R. Hodges and M. A. Rhian, *Exptl. Med. and Surg.* **21**, in press.
6. J. Keppie, H. Smith, P. W. Harris-Smith, *Brit. J. Exptl. Pathol.* **36**, 315 (1955).
7. P. Malek, J. Kolc, F. Zak, *Zentr. Bakteriol. Parasitenk. Abt. I Orig.* **174**, 94 (1960).
8. C. B. Thorne, D. M. Molnar, R. E. Strange, *J. Bacteriol.* **79**, 450 (1960).
9. E. N. Shliakhou, *J. Microbiol. Epidemiol. Immunobiol. (U.S.S.R.)* **28**, 748 (1957), English translation (Pergamon, New York).
10. S. H. Plotkin, P. S. Brachman, N. Utell, *Am. J. Med.* **29**, 992 (1960).

13 September 1962

Genetic and Environmental Variation: Effect on Pigments of Selected Maize Mutants

Abstract. The chloroplast pigments of three "pastel" mutants of maize were found to vary with temperature as well as with genotype.

It has long been known that chlorophylls, carotenes, and xanthophylls are universally present in chloroplasts and also in the purple and green bacteria (*1*). Many mutations have occurred that affect the chloroplast pigments. They vary in effect from a complete lack of all of the pigments in question to a slight change in the structure or concentration of one of the pigments. In the latter class are some which appear different under different temperature conditions because of variations in the pigment concentrations. That is, the mutants are not only of a phenotype intermediate between the albino and the normal, but their phenotypes can be varied experimentally when the temperature of the environment is controlled.

For maize, Phinney has described the appearance of certain virescents after various temperature treatments (*2*), but, with one exception (*3*), no attempt has been made heretofore to describe chemically the phenotype differences between the normal plants and intermediate mutants under various treatments.

In this study, three pastel mutants which were sensitive to temperature and the offspring of two of these with allelic albino mutants were studied under conditions of controlled light and temperature. Pastel mutants are not only pale green in phenotype, but have a light yellow or white endosperm which is distinct from the yellow of the normal siblings.

The mutants used in the experiment were: pas_{8686} (pastel), its allele w_3 (white), on chromosome 2; pas_{4889}, its allele vp_9 (viviparous), on chromosome 7; and pas_{8549}, on chromosome 6. $Pastel_{8549}$ had been previously studied by Robertson and Anderson (*3*) under other conditions of light. This genetic material was obtained from the stocks maintained at Iowa State University. The mutants w_3 and vp_9 are viviparous albinos (*4*); pas_{8686} and pas_{4089} are tem-

Table 1. Pigment contents of normal (+) and mutant (m) corn seedlings as influenced by temperature.

Seedling	Temp. (°C)	Chlorophyll (mg/g fresh wt.)	m/+ (%)	Carotene (mg/g fresh wt.)	m/+ (%)	Xanthophyll (mg/g fresh wt.)	m/+ (%)
				pas_{8549}			
+	22	2.551	68.1	0.0531	92.1	0.0556	81.5
m		1.737		.0489		.0453	
+	37	2.382	13.9	.0777	66.7	.0622	22.5
m		0.330		.0517		.0140	
				pas_{4889}			
+	22	2.746	19.5	.0746	20.5	.0583	50.9
m		0.536		.0153		.0297	
+	37	2.717	43.9	.0755	51.8	.0731	33.2
m		1.193		.0391		.0243	
				vp_9/pas_{4889}			
+	22	2.575	7.9	.0809	10.1	.0960	15.3
m		0.203		.0082		.0147	
+	37	2.577	16.7	.0873	13.9	.0586	43.3
m		0.430		.0121		.0254	
				pas_{8686}			
+	22	2.477	11.1	.0658	7.9	.0407	45.0
m		0.275		.0052		.0183	
+	37	2.870	59.6	.0787	61.4	.0407	144.7
m		1.710		.0483		.0589	
				w_3/pas_{8686}			
+	22	3.546	2.8	.1105	2.2	.0780	12.8
m		0.100		.0024		.0100	
+	37	2.419	22.8	.0903	14.2	.0670	45.7
m		0.552		.0128		.0307	