extracted collagen from the skins of control and lathyritic rats provided evidence that the excess soluble collagen molecules which accumulate in the tissues of lathyritic animals are intrinsically normal. But the lathyritic molecules are dramatically deficient in aldehyde content. Thus it appears that lathyrogens exert their effect on connective tissues by causing an inhibition of the oxidative deamination which appears to precede covalent crosslink formation in collagen and elastin.

The authors express appreciation to Dr. Nils Eriksen for valuable assistance during some phases of this work and to Miss Karen Wick for expert technical assistance.

1. Selye, H., Rev. Canad. Biol., 1957, v16, 1.
2. Levene, C. I., Gross, J., J. Exp. Med., 1959, v110, 771.
3. Martin, G. R., Piez, K. A., Lewis, M. S., Biochim. Biophys. Acta, 1963, v69, 472.
4. Smiley, J. D., Yeager, H., Ziff, M., J. Exp. Med., 1962, v116, 45.
5. Tanzer, M. L., Gross, J., ibid., 1964, v119, 274.
6. Page, R. C., Benditt, E. P., Lab. Invest., Nov. 1966, v15, 1643.
7. Bornstein, P., Kang, A. H., Piez, K. A., Proc. Nat. Acad. Sci., 1966, v55, 417.
8. Partridge, S. M., Elsden, D. F., Thomas, J., Dorfman, A., Telser, A., Pei-Lee Ho, Biochem. J., 1964, v93, 30c.
9. Franbzlau, C., Sinex, F. M., Faris, B., Lampidis, R., Biochem. Biophys. Res. Comm., 1965, v21, 575.
10. Benditt, E. P., Eriksen, N., Arch. Path., 1964, v78, 325.
11. Piez, K. A., Eigner, E. A., Lewis, M. S., Biochemistry, 1963, v2, 58.
12. Bergman, I., Loxley, R., Anal. Chem., 1963, v35, 1961.
13. Paz, M. A., Blumenfeld, O. O., Rojkina, M., Henson, E., Furfine, C., Gallop, P. M., Arch. Biochem. & Biophys., 1965, v109, 548.
14. Miller, E. J., Fullmer, H. M., J. Exp. Med., 1966, v123, 1097.
15. Nantö, V., Maatela, J., Kulonen, E., Acta Chim. Scand., 1963, v17, 1604.
16. Gallop, P. M., Nature, 1966, v209, 73.
17. Petruska, J., Hodge, A., Proc. Nat. Acad. Sci., 1964, v51, 871.
18. Ingram, V. M., Nature, 1956, v178, 792.
19. Huisman, T. H. J., Clin. Chimica Acta, 1958, v3, 201.
20. Miller, E. J., Martin, G. R., Mecca, C. E., Piez, K. A., J. Biol. Chem., 1965, v240, 3623.

Received October 28, 1966. P.S.E.B.M., 1967, v124.

Amino Acid Composition of Three Immunological Types of Foot-and-Mouth Disease Virus. (31765)

HOWARD L. BACHRACH AND JEROME POLATNICK
Plum Island Animal Disease Laboratory, Animal Disease and Parasite Research Division, Agricultural Research Service, U.S. Dept. of Agriculture, Greenport, L.I., N.Y.

The amino acid compositions of only a few animal viruses have been determined, *e.g.*, polio, influenza, Shope papilloma and EMC viruses(1). The main reason for this is the difficulty in obtaining animal viruses in a state of purity in quantities adequate for meaningful analyses. We have described methods that yield essentially pure foot-and-mouth disease virus (FMDV) in milligram amounts from calf-kidney (CK) and baby-hamster kidney (BHK) cell cultures(2,3). This report contains the results of amino acid analysis of 3 immunological types of FMDV purified from tissue cultures.

Materials and methods. Virus. The immunological types of FMDV were type A, strain 119; type O, strain 9; and type C, strain 3. Type A_{119} virus was obtained from the Research Institute, Pirbright, England, as infectious bovine-tongue epithelial tissue. One portion was passed at Plum Island once in cattle, once in mice, and thereafter, approximately 150 times in primary CK cultures over a 12-year period. Large quantities of this virus were subsequently produced by single passages in uncloned BHK cell cultures in 2-liter roller bottles(3). Another portion of the bovine virus remained frozen for 12 years and was then passed 8 times in Povitsky-bottle cultures of CK cells. Hereafter, it is desig-

nated as A_{119}^{CK} virus to distinguish it from the type A_{119} virus with final passage in BHK cells. Types O_9 and C_3 virus derived from cattle in Argentina were grown in sufficient quantity by 2 and 4 passages, respectively, in BHK cell cultures in roller bottles. Essentially pure virus was obtained from culture fluids by a procedure(2) which consists of alcohol precipitation, organic solvent extractions, CsCl density gradient centrifugation, centrifugation through an organic solvent mixture into CsCl, and dialysis against 0.05 M sodium-phosphate buffer at pH 7.5. Purified virus contained 10^{10} to 10^{11} plaque-forming units of virus per ml. By spectral analysis, using an extinction coefficient $E^{1\%}_{259 m\mu}$ of 76.0(2), virus concentrations ranged from 2 to 10 mg/ml. Confirmation of the type and strain of the purified virus was obtained by cross neutralization and agar-gel precipitin tests.

Digestion of virus. Since preparations of soluble virus protein sufficiently free of ribonucleic acid (RNA) were not obtainable, amino acid analyses were carried out on virus *per se*. This necessitated the development of a correction factor for glycine that is described later. The hydrolysis procedure was relatively standard(8): 0.5 ml of virus was mixed with 9.5 ml constant-boiling, 5.54 N HCl. The mixtures were cooled, evacuated to 50 μ Hg, flushed 3 times with N_2, evacuated again and hydrolyzed at 110°C for 41 hours. Hydrolysates were filtered quantitatively through sintered glass and flash evaporated twice to dryness. Dry samples were resuspended in 2 ml of 0.1 N sodium-citrate buffer, pH 2.2, and 0.9 ml was applied to each "acidic and basic" chromatographic column.

Preliminary to comparative analyses of the different types of virus, the degree of hydrolysis of type A_{119} virus was examined at 16.5, 20, 41, 53 and 72 hours. Concentrations of most of the amino acids reached a maximum by 41 hours, and this hydrolysis time was used in the remaining experiments. However, serine, threonine, tyrosine, phenylalanine and proline showed linear decreases in concentration after 22 hours due to hydrolytic decomposition. Correction factors for these amino acids were obtained by extrapolation to zero time hydrolysis. The reported values for these amino acids at 41 hours have thus been increased by 15.3, 7.5, 6.0, 4.4 and 3.6%, respectively. Ammonia increased with prolonged digestion because of its formation from the nucleic acid bases. This source of ammonia precluded the determination of protein amide nitrogen. Glycine increased similarly by formation from nucleic acid bases(4). Constitutive glycine was taken as the difference between glycine obtained during 41 hours digestion of virus and viral RNA. Ribonucleic acid for this purpose was prepared from type C_3 virus by 6 extractions with water-saturated phenol containing 0.01% ethylenediaminetetraacetate followed by 5 extractions with peroxide-free ether. Residual ether was bubbled off with nitrogen. The RNA was dialyzed against 0.05 M sodium phosphate at pH 7.5 before determining concentration from absorbency at 258 mμ. An extinction coefficient, $E^{1\%}$, of 220 was used(2). Duplicate samples, each containing 1.32 mg of RNA, were hydrolyzed in 5.54 N HCl at 110°C for 41 hours and analyzed for amino acid content. The glycine concentration was 0.30 μM. Minute amounts of aspartic acid, threonine, serine, glutamic acid, alanine and valine were also present, indicating that a trace amount of protein remained in the RNA solution, after 6 phenol extractions.

Tryptophan. Tryptophan, which is destroyed by acid digestion, was determined by its absorbence at 590 mμ after reaction of low-ionic strength degraded virus(15) with dimethylaminobenzaldehyde and sodium nitrite in H_2SO_4 solution(5). Since standard curves for tryptophan were identical in the presence and absence of purified viral RNA, this method(5) is valid for RNA viruses. The standard curves were linear down to 1 μg tryptophan per sample.

Cystine-cysteine ratio. Total cystine plus cysteine in type A_{119} virus was measured by oxidation to cysteic acid with performic acid (6). Two milliliters of performic acid were added to 1.51 mg of dry virus and held at 0°C for 2.5 hours. After dilution with 22.5 ml of ice water, the reaction mixture was flash evaporated at 20°C to 1 ml and frozen-

dried. The dry product was suspended in 0.5 ml water and digested with 9.5 ml constant boiling HCl as described above prior to chromatographic analysis for cysteic acid. Cysteine was determined as S-carboxymethylcysteine(7) in the presence of 6 M urea as denaturing agent. Six-tenths ml of 5% ICH_2COOH, 2.2 ml of freshly prepared 11 M urea and 0.82 ml (1.51 mg) of type A_{119} virus were agitated at room temperature for 30 minutes at pH 9.0. After exhaustive dialysis against water, the product was frozen-dried, resuspended in 0.5 ml of water and digested with HCl as described above. The hydrolysate was analyzed on the "acid" chromatographic column for its content of S-carboxymethylcysteine.

Chromatography. Hydrolysis products were analyzed by the Spackman accelerated modification of the Stein-Moore procedure(8, 9) in a Phoenix Model K-8000B amino acid analyzer. Its precision bored glass columns were packed with sulfonated styrene-8% divinyl benzene copolymerized resin. The short "basic amino acid" column measured 12 × 0.9 cm diameter; the longer "acidic amino acid" column, 50 × 1.2 cm diameter. The amount of hydrolyzed virus which was applied to columns for determining amino acid compositions ranged from 342 to 873 μg. In experiments to determine the cystine-cysteine ratio, 1510 μg samples were used. Approximately 0.68 of the weight of FMDV is protein(2). Areas under the recorded peaks for amino acid-ninhydrin reaction products were integrated by the height-×-width method. The 440 mμ absorbence peak was used for the determination of proline; 570 mμ absorbence was used for all other amino acids. The calibration test mixture (Phoenix-K18) contained 17 amino acids at 2.50 ± 0.05 μM.

Statistical evaluations. Statistical comparisons were made of 2 analyses of one virus type with 2 analyses of another type. For 1 and 36 degrees of freedom, f_n values greater than 4.11 and 7.39 indicated differences in a specific amino acid at the 0.05 and 0.01 levels of significance, respectively(10).

Results. Amino acid analyses on 3 immunological types of FMDV are presented in Table I. The data, in moles per 100 moles of amino acids, are for 2 runs on each virus type. Independent preparations of purified virus were used for each run on types A_{119}^{CK} and O_9 virus. Those for type A_{119} were carried out on a pool of 3 independent preparations. A single preparation of type C_3 virus served for both of its analyses. Despite variability in the total recovery of amino acids, there is good agreement between the 2 runs for each virus type except for half-cystine and methionine values. This difficulty is considered due to the small amounts of these 2 amino acids in the virus. Attempts to obtain more precise values for them by chromatographing larger quantities of hydrolyzed virus on the 1.2 cm diameter column resulted in poor resolution of the amino acids.

It may be noted that A_{119}^{CK} and A_{119} viruses, which differ only in post-cattle passage histories, appear to possess differences in their content of 3 amino acids—glycine, tryptophan and tyrosine. In accordance with immunological differences, types A_{119}^{CK}, O_9 and C_3 viruses which had been passed only a few times in cultures showed differences in composition. Type A_{119}^{CK} virus contained more histidine and lysine but less alanine, half-cystine, leucine and tryptophan than type O_9. In comparison to type C_3, type A_{119}^{CK} contained more glycine, isoleucine, lysine, phenylalanine and serine but less alanine, proline, threonine and valine. A further test showed types O_9 and C_3 to differ in 11 amino acids.

An attempt was made to determine the proportion of cystine to cysteine in A_{119} virus. Approximately 0.034 μM of cysteic acid was obtained after performic acid oxidation of 1.51 mg of virus compared with 0.010 μM of S-carboxymethylcysteine by alkylation of an equal amount of virus in 6 M urea. Provided that all the —SH groups of the virus protein reacted with iodoacetate, it appears that there are approximately equal numbers of disulfide and sulfhydril groups in the virus. However, from the electrophoretic behavior of the protein of urea-degraded FMDV on acrylamide gels, the efficacy of urea in dispersing the protein and exposing buried sulfhydril

TABLE I. Amino Acid Composition of Three Types of Foot-and-Mouth Disease Virus.

Amino acid*	Virus type†				Significance‡		
	A_{119}^{CK}	A_{119}	O_9	C_3	A_{119}	A_{119}^{CK} *vs* O_9	A_{119}^{CK} *vs* C_3
	moles per 100 moles amino acids						
Ala	8.38 ± .07	8.31 ± .04	9.49 ± .19	10.24 ± .06		++	++
Arg	3.86 ± .28	3.91 ± .09	3.32 ± .14	4.24 ± .15			
Asp	10.07 ± .08	10.38 ± .01	10.26 ± .02	10.09 ± .13			
Cys	.74 ± .35	.53 ± .03	1.25 ± .22	.63 ± .24		+	
Glu	8.59 ± .10	8.76 ± .31	8.83 ± .12	8.79 ± .08			
Gly	7.05 ± .02	7.81 ± .26	7.29 ± .24	6.69 ± .05	++		++
His	3.29 ± .10	3.27 ± .13	2.62 ± .05	3.25 ± .05		+	
Ile	3.38 ± .26	3.57 ± .02	3.49 ± .09	2.83 ± .04			++
Leu	7.59 ± .20	7.34 ± .11	8.31 ± .02	7.55 ± .06		++	
Lys	4.23 ± .06	4.59 ± .17	3.71 ± .19	3.68 ± .06		++	++
Met	1.31 ± .44	1.82 ± .20	1.72 ± .19	1.54 ± .34			
Phe	4.13 ± .04	3.73 ± .03	4.19 ± .14	3.16 ± .01			++
Pro	5.82 ± .18	5.44 ± .17	5.74 ± .97	5.93 ± .06			+
Ser	6.93 ± .05	7.00 ± .06	6.72 ± .27	6.04 ± .09			++
Thr	10.89 ± .14	10.91 ± .09	10.92 ± .02	11.76 ± .25			++
Trp	1.99 ± .15	.88 ± .03	1.64 ± .17	.94 ± .00	++	+	
Tyr	5.52 ± .30	4.76 ± .16	4.81 ± .11	5.01 ± .07	++		
Val	6.99 ± .17	6.94 ± .05	7.37 ± .11	7.66 ± .21			++
% Recovery§	57, 66	86, 94	54, 81	78, 79			

* Abbreviations from(11); protein amides not determinable on virions.

† For passage histories refer to *Materials and methods*; errors given as average deviations indicate the agreement between the 2 runs on a virus type.

‡ F-test comparison of both runs of one type with both runs of another; +, significantly different at 0.05 level; ++, at 0.01 level.

§ Weight amino acid residues recovered × 100 divided by weight of protein sample, *i.e.*, virus weight × 0.68. Values are for the 2 runs on each virus.

groups is questionable (Vande Woude and Bachrach, in preparation).

Discussion. As seen in Table I, FMDV contains 18 of the commonly occurring amino acids and no unusual ones. In accord with findings for most other plant and animal viruses(1), sulfur-containing amino acids and tryptophan are present in minimal amounts in FMDV protein. Also, the protein appears to contain 1.7 times as many acidic as basic amino acid residues. Its content alone of aspartic and glutamic acid residues is approximately 19%.

Several studies on A_{119} and A_{119}^{CK} viruses support the finding that these 2 viruses differ in amino acid composition. First, a marked decrease in virulence for cattle occurred prior to the 129th serial passage of A_{119} virus in primary CK cultures(12). Second, as determined by absorbence-temperature profiles, purified A_{119}^{CK} virus degrades into protein and RNA at 35°C in 0.01 M sodium phosphate at pH 7.5 (ref. 13, Fig. 5, CK_L virus). By comparison, A_{119} virus degrades at 51-53°C whether or not it is produced in primary CK cells(14), uncloned BHK(15) or clone-13-derived BHK cells (ref. 13, Fig. 5, CK_H-BHK_S^{13} virus). Third, the electrophoretic mobilities of A_{119} (from BHK^{13} cells) and A_{119}^{CK} viruses are significantly different, being 2.92×10^{-5} and 3.28×10^{-5} cm²/volt·sec, respectively(13). Thus, long adaptation to tissue culture produced a considerably more heat-stable virion with less net negative charge. The decrease in mobility of A_{119} virus had, moreover, occurred prior to its final single passage in BHK^{13} cells (ref. 13, Table I, run nos. 5 and 6).

The above findings indicate that the process of translation of viral RNA into coat protein is not altered on passing from primary CK cells to BHK cells. Rather, A_{119} and A_{119}^{CK} viruses appear to have become genotypically different either by selection or mutation during post-cattle passages (see *Materials and methods*) in CK cells. The observed phenotypic differences in degradation temperature, electrophoretic mobility and amino acid com-

position must be expressions of this basic genetic difference. Since both isolates are still of the A type, the change is very likely not in the viral cistron which codes for the immunogenic peptide sequence on the outside of the coat protein. Although the amino acids involved must be sufficiently exposed to affect electrophoretic mobility, the increased temperature stability of the highly adapted A_{119} virus indicates a better chemical bonding between buried protein and that portion of the viral RNA which appears to be laced through it(14). Similarly, its lower mobility indicates either better shielding of the negatively charged viral RNA or a net gain of acidic amino avids. The latter, however, does not appear to have occurred (Table I).

The different amounts of 3 amino acids in A_{119} and A_{119}^{CK} viruses could be attributed to single and adjacent double base mutations in viral RNA. Replacement of tryptophan by glycine would result from a codon change of UGG to GGG. Glycine would replace tyrosine by a change of UAU to GGU or of UAC to GGC. Amino acid sequence studies on tryptophan synthetase mutants have, in fact, indicated that a single amino acid replacement can result from a change in two adjacent bases(16). The mutant occurred rarely, however, and did not revert to the wild-type.

Summary. There were significant differences in several of the 18 amino acids of foot-and-mouth disease viruses—immunological types A_{119}^{CK}, O_9 and C_3—which had been passed only a few times in tissue cultures. In addition, low passage A_{119}^{CK} virus differed from high passage A_{119} virus in the 3 amino acids—glycine, tryptophan and tyrosine. This difference was correlated with previous findings concerning virulence, electrophoretic mobilities and degradation temperatures.

We wish to thank Drs. K. Cowan and G. E. Cottral for virus-typing tests, Mr. R. P. Lehmann (Biometrical Services, Agricultural Research Service, Beltsville, Md.) for recommending statistical procedures, and Mr. Karl Axelson for technical assistance.

1. Knight, C. A., Protoplasmatologia, Hundbuch der Protoplasmaforsch., IV, Springer-Verlag, Vienna, 1963, p43.

2. Bachrach, H. L., Trautman, R., Breese, S. S., Jr., Am. J. Vet. Res., 1964, v25, 333.

3. Polatnick, J., Bachrach, H. L., Appl. Microbiol., 1964, v12, 368.

4. Spahr, P. F., J. Mol. Biol., 1962, v4, 395.

5. Spies, J. R., Chambers, D. C., Anal. Chem., 1949, v21, 1249.

6. Hirs, C. H. W., J. Biol. Chem., 1956, v219, 611.

7. Moore, S., Cole, R. D., Gundlach, H. G., Stein, W. H., Proc. 4th Int. Cong. Biochem., Vienna, 1958, p52.

8. Spackman, D. H., Stein, W. H., Moore, S., Anal. Chem., 1958, v30, 1190.

9. Spackman, D. H., Fed. Proc. (Abst.), 1963, v22, 244.

10. Snedecor, G. W., Statistical Methods, 5th ed., Iowa State Univ. Press, Ames, 1956.

11. Cohn, W. E., IEG, 7, 1966, Memo 399.

12. Cottral, G. E., Patty, R. E., Gailiunas, P., Scott, F. W., Arch. ges. Virusforsch., 1966, v18, 276.

13. Matheka, H. D., Bachrach, H. L., Trautman, R., Z. Naturforsch., 1966, v21b, 774, in English.

14. Bachrach, H. L., J. Mol. Biol., 1964, v8, 348.

15. ———, Virology, 1965, v25, 532.

16. Crick, F. H. C., Sci. Am., 1966, v215, 55.

Received October 31, 1966. P.S.E.B.M., 1967, v124.