THE FREE AND PROTEIN-BOUND AMINO ACIDS OF CERTAIN NICOTIANA SPECIES AND HYBRIDS[1,2]

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INTRODUCTION

Many of the nearly 60 wild species of *Nicotiana* can be hybridized with tobacco (*N. tabacum* L.). These wild species constitute a potential reservoir for the transference of quality constituents in the same way that several have been used as sources of resistance to tobacco diseases.

In 1963, Weybrew and Mann (9) published comparative analytical data on certain *Nicotiana* species and their F_1 hybrids with *N. tabacum;* rather large quantitative differences were demonstrated for many of the more than 70 constituents included. But largely because efficient and reliable analytical methods were not then available, this earlier survey contained only limited data on the amino acids. The present paper supplements the original report by comparing the amino acid compositions, both free and protein-bound, of new samples of these same genetic stocks.

MATERIALS AND METHODS

Plant Samples. Plants of the subject lines were grown, like flue-cured tobacco, in three replications of a randomized block experiment at the Central Crops Research Station, Clayton, N. C., in 1964. Entries included *N. tabacum* (two flue-cured varieties, SC58 and Coker 139), *N. sylvestris, N. tomentosiformis, N. otophora, N. glauca* (two accessions, yellow or purple-flowered), *N. glutinosa,* and all 12 F_1 hybrids combinations between each of the tobacco varieties and all of the other species.

As best maturity could be judged, leaves were harvested when ripe and were flue-cured, using the progress of the true tobaccos as the guide for regulating the curing schedule. Whole-plant laminar samples of the cured materials were re-dried, ground in a Wiley mill, and stored in sealed bottles at about 25° C until analyzed.

Pedigrees, cultural details, and agronomic performances of these 20 entries have been published elsewhere (Matzinger and Wernsman, 5).

Preparation of Extracts. (1) *Free Amino Acids.* Free amino acids were extracted from the pulverized samples with 1% HCl in the proportion 1:20 (w/v) as described by Burde, *et al.* (1). To 2.000 gm of the powdered sample contained in a 250-ml glass-stoppered Erlenmeyer flask was added 40 ml of 1% HCl. After shaking for one hour on a Burrell wrist-action shaker,

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Table 1. Average "free" amino acids of certain Nicotiana species and hybrids.

Constituent	Parental Nicotiana Species							F_1 Hybrids[a]					L.S.D.		CV
	N. tabacum C139	N. tabacum SC58	sylvestris	tom · fms	otophora	glauca[b]	glutinosa	tab x syl	tab x tom	tab x oto	tab x glo	tab x glu	.05	.01	(%)
	Micrograms per gram, (Average of 3 replications)														
Lysine	22	48	112	143	241	116	285	54	124	86	109	171	57	77	29
Histidine	157	207	509	227	236	972	981	343	660	328	694	645	238	320	26
Ammonia	440	653	1150	968	654	1380	847	746	909	830	1630	996	308	414	18
Arginine	10	10	62	197	97	78	116	54	125	92	126	170	225	NS	75
L_{x_1} (Cysteic Acid)	179	169	139	232	174	124	76	157	203	186	202	153	58	78	20
Taurine, (L_a)	713	1120	370	494	444	441	58	875	989	855	300	821	174	235	16
L_{x_2} (Unknown)	239	191	36	17	32	23	41	183	54	117	16	137			
Hydroxyproline	629	268	—	—	—	—	—	28	3	33	—	—			
Methionine-S-oxide	1430	1120	228	304	356	361	255	1070	628	738	237	542	232	311	25
Aspartic Acid	296	261	494	891	1040	1610	1140	493	994	582	1360	550	349	469	23
STAG	897	1480	4980	5600	4710	9020	9830	2340	5400	3320	9730	4040	2320	3120	24
Glutamic Acid	196	335	363	215	240	926	878	296	339	255	401	499	135	182	19
Proline	3210	3140	799	1410	3100	1470	445	3820	3580	3990	1010	7560	1020	1370	21
α-Aminoadipic Acid	435	378	51	42	95	73	101	272	182	301	41	197	147	198	42
Glycine	56	53	105	80	81	141	150	61	83	69	123	90	22	29	13
Alanine	443	508	471	497	823	826	1320	860	669	744	871	1180	247	332	18
L_2 (Unknown)	567	493	9	†	†	—	—	307	48	230	†	98			
Cystine	—	—	91	47	†	77	90	—	†	†	†	†			
Valine	59	77	368	251	141	134	1420	134	220	138	130	209	183	246	49
Isoleucine	34	41	142	75	46	89	596	63	78	50	83	79	78	105	47
Leucine	10	11	92	81	66	69	527	30	61	42	58	38	82	111	66
Tyrosine	513	405	184	134	181	258	252	348	289	315	114	256	75	101	18
Phenylalanine	154	223	648	654	336	421	1380	378	789	450	524	839	326	439	35
Glucosamine	443	517	1000	235	401	241	—	723	596	654	304	1350	345	466	36
Homocystine	81	67	†	7	†	—	—	57	23	40	†	20			
Galactosamine	173	247	81	105	141	121	—	316	307	320	114	449	139	188	38
β-Alanine	—	—	170	94	124	172	439	—	p	p	143	p			
L_{x_3} (Unknown)	252	305	110	145	175	127	—	293	278	326	126	297	98	133	29
L_{x_4} (Unknown)	105	126	19	†	†	†	—	89	56	68	†	57			
γ-Aminobutyric Acid	104	158	303	434	467	958	477	236	336	286	477	369	149	200	22

See footnotes below Table 1a.

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Table 1a. Means and statistical significance of certain contrasts: Free amino acids (μg/gm).

Constituent	Among Parents	Among Hybrids	Weighted Means of Parents	Weighted Means of Hybrids	(F)	F_2 Hybrids of C139	F_2 Hybrids of SC58	(F)	Among Species in Hybrids	Among tab x spec. in Hybrids
Lysine	**	**	102	109	NS	91	126	**	**	**
Histidine	**	**	416	561	**	555	566	NS	**	**
Ammonia	**	**	805	1122	**	1070	1175	NS	**	NS
Arginine	**	NS	144	116	NS	98	134	NS	NS	NS
L_1 (Cysteic Acid)	**	**	159	183	*	186	181	NS	**	NS
Taurine, (L_8)	**	**	646	691	NS	721	659	NS	**	NS
L_4, (Unknown)										
Hydroxyproline										
Methionine-S-oxide	**	**	792	576	**	704	448	**	**	**
Aspartic Acid	**	**	704	891	**	816	966	*	**	NS
STAG	**	**	4190	5760	**	4840	6690	**	**	NS
Glutamic Acid	**	**	428	365	*	334	396	*	**	NS
Proline	**	**	2310	3500	**	3490	3500	NS	**	**
α-Aminoadipic Acid	**	**	240	172	**	215	129	**	**	NS
Glycine	**	**	85	92	NS	84	99	**	**	*
Alanine	**	**	635	865	**	831	900	NS	**	*
L_7, (Unknown)										
Cystine										
Valine	**	NS	238	160	*	148	172	NS	NS	NS
Isoleucine	**	NS	105	73	*	69	76	NS	NS	NS
Leucine	**	NS	80	48	*	44	51	NS	NS	NS
Tyrosine	**	**	335	239	**	280	198	**	**	NS
Phenylalanine	**	**	416	584	**	509	660	**	**	NS
Glucosamine	**	**	418	655	NS	560	735	*	**	**
Homocystine										
Galactosamine	*	**	153	270	**	285	254	NS	**	**
β-Alanine										
L_{11}, (Unknown)	**	**	196	242	*	263	219	*	**	NS
L_{12}, (Unknown)										
γ-Aminobutyric Acid	**	**	365	363	NS	331	396	*	**	NS

[a] *Hybrids involving C139 and SC58 have been averaged together as have also those of the two glaucas.*
[b] *Average of the "yellow" and "purple" varieties.*

P = present but not resolved.
t = trace.
— = not detected.

** significant at P .05*
*** significant at P .01*
NS not significant (.05)

the extract was stored at 4° C in a refrigerator overnight. The contents of the flask were then transferred to a 50-ml polyethylene centrifuge tube and centrifuged at 2500 rpm (~1340 x *g*) for 15 minutes. The supernatant was decanted through an E & D No. 613 filter. (The tissue residue was retained for the preparation of hydrolysates, as will be described subsequently.)

Crude extracts were deproteinized with picric acid as suggested by Hamilton (4). A 15-ml aliquot of the filtered extract was pipetted into a 50-ml polyethylene centrifuge tube, and 15 ml of 1% picric acid was added. After mixing, the tube was immersed in a boiling water bath for 30 minutes, then centrifuged for eight minutes at 2000 rpm (~ 885 x *g*). The supernatant extract was carefully decanted and adjusted back to 30 ml with 1% HCl. Excess picric acid was removed by adsorption on Dowex 2-X8 resin. Finally the clarified extract was filtered through a coarse sintered-glass funnel.

Because amides, particularly asparagine, in these extracts tend to hydrolyze slowly even when stored at 4° C, extraction did not precede analysis by more than a day or two.

(2) *Protein Hydrolysates.* The tubes containing the tissue residues from the above extractions were inverted and allowed to drain for 10 minutes. The plant material was resuspended in another 40-ml of 1% HCl, allowed to stand for 24 hours, then centrifuged. The supernatant washing was decanted and discarded, the tube was drained, then resuspended and washed a second time. Analysis of the washings demonstrated that the two serial washings were effective in ridding the plant material of free amino acids.

The washed and drained residue was transferred to an aluminum-foil dish, dried in a forced-air oven at 101° C for four hours, then repulverized in an agate mortar.

A modification of the procedure described by Spackman (8) was used for the hydrolysis of the *Nicotiana* proteins. Preliminary analyses of the extracted materials by the method of McKenzie and Wallace (6) had indicated that a 40-mg sample would contain the appropriate quantity of protein-N. The accurately weighed sample was carefully transferred into the bottom of a lipless Pyrex test tube, 16 x 150 mm. One ml of 6N HCl (prepared by diluting fresh concentrated HCl, Analytical Reagent grade, with an equal volume of demineralized water) was added, washing down the walls of the tube. With the tube in the upright position and using an oxygen-gas flame, the tube was carefully constricted to an inside diameter of 1 mm or less about 3 cm below the open end. The contents of the tube were frozen by immersion in an alcohol-Dry Ice bath for about five minutes then, still immersed, a vacuum pump capable of pulling a vacuum of 0.1 mm or better was attached. While under vacuum, the tube was removed from the cold bath and quickly sealed off in the flame.

Hydrolysis of the protein was effected by placing the sealed ampoules in a forced-air oven at 110° C for 22 hours. The sealed hydrolysates are stable indefinitely but were stored in a refrigerator at 4° C until scheduled for analysis.

Just prior to the analysis, the ampoule was broken and the HCl was evaporated completely under vacuum. Exactly 5 ml of 0.20 N citrate buffer, pH 2.2 ± .03, was measured into the ampoule which was then immersed in a boiling water bath to insure solution of the amino acids. Finally, the hydrolysate solution was filtered into a small labeled vial.

Under conditions of the hydrolysis, some cystine may be reduced to cysteine and this compound elutes from the analytical column coincident with proline. Any cysteine artifact could be reconverted into cystine if the residue after evaporation were to be taken up in 1 ml of phosphate buffer at pH 6.5 and allowed to stand for four hours at room temperature before adjusting to pH 2.2 wtih citrate. Several pairs of hydrolysate residues were either phosphated or handled by the normal procedure described above and then analyzed. Neither treatment contained more than traces of cystine and the proline peaks were identical within experimental error. It was concluded therefrom, the cystine was such a minor constituent of *Nicotiana* that the phosphating precaution was not necessary.

Analysis. The separation and analysis of the individ-

Table 2. Differences in certain "free" amino acids in related inter-specific combinations.

Constituent	F_1 hybrid, tab. x (species) syl.	tom.	oto.	gla.	glu.
	Average difference, SC58 F_1 minus C139 F_1, μg/gm				
Lysine	4	— 32	61	36	143
Histidine	71	—520	17	192	308
Methionine-S-oxide	—568	—193	—230	— 62	—484
Proline	80	—1121	—817	213	1717
Glycine	3	— 3	11	50	34
Alanine	— 16	—202	32	509	94
Glucosamine	332	14	—179	— 36	830
Galactosamine	160	— 42	—211	—151	60

ual amino acids was accomplished on a Spinco Amino Acid Analyzer, Model 120B (Spinco Division, Beckman Instruments Inc., Palo Alto, California) operated at 55° C with flowrates of 68 and 34 ml/hr of buffer and ninhydrin respectively. The basic amino acids were separated on a column (0.9 x 7 cm) of AA-27 spherical resin, eluting with 0.35 N citrate buffer at pH 5.28 ± .02. Acidic and neutral constituents (in a second aliquot) were eluted sequentially from an AA-15 resin column (0.9 x 57 cm), eluting first with 0.20 N citrate buffer at pH 3.28 ± .01 and abruptly switched to 0.20 N citrate buffer, pH 4.25 ± .02 at 134 minutes into the analysis.

The appropriate analytical aliquot was 2 ml of the free amino acid extract (equivalent to 50 mg of sample) or 1 ml of hydrolysate (equal to 8 mg of extracted residue when the weighed amount had been 40 mg). One complete free amino acid analysis required 6.5 hours, while the separation of the component amino acids in the hydrolysates could be completed in four hours.

Analytical chromatograms of the 1% HCl extracts of tobacco show several peaks in addition to the true α-amino acids (**Figure 1**); thus, in the present context, a free amino acid is defined as any compound (a) that is extractable with 1% HCl, (b) that is not precipitated with picric acid, and (c) that gives a color with ninhydrin. A few of these other compounds have been tentatively identified, principally by the technique of co-elution with authentic specimens but supported by their relative 440/570 mμ absorptions (10). Several are still "unknowns." The elution sequence and the key to peak identifications are iven in the caption to **Figure 1.**

Factors for the quantification of peak areas were obtained from repetitive analyses of mixtures containing 0.5 μmole quantities of the pure compounds. Concentrations of unknown constituents have been approximated through the use of an "average" factor and an "average" molecular weight.

RESULTS

Free Amino Acids. Concentrations of the free amino acids for each of the species parents (including both varieties of *N. tabacum*) and for each hybrid combination between *N. tabacum* and each of the other species are presented in **Table 1.** The two ascessions of *N. glauca* ("yellow" and "purple") have been averaged together in this table as have also all F_1 hybrids having a common parent. The L.S.D. values included in the table are those that were generated for the intercomparisons among means containing three observations and thus would apply to the two *N. tabacum* varieties and to all of the species except *N. glauca.* The data for *N. glauca* and for each of the F_1 hybrids contain at least six observations per mean (the *N. tabacum* x *N. glauca* hybrid contains 12) and accordingly, the appropriate L.S.D's would be somewhat smaller. However, conclusions based on the broader application of the given L.S.Ds would be on the side of conservatism.

Statistical analyses on the free amino acid data and tests of significance are summarized in **Table 1a.** For seven constituents for which the data are incomplete either due to the levels being too low for valid estimations or because of poor analytical resolution, statistical analyses were not performed.

(1) *Among Parental Species.* Significant differences existed among species for all constituents (Table 1a, Column 1). *N. tabacum* differed, both quantitatively and qualitatively, from the other species for a number of constituents. Hydroxyproline, L_7, homocystine, and L_{12} were present in both varieties of *N. tabacum* whereas the other species contained no more than traces of these compounds. Conversely, cystine and β-alanine were not

Table 3. Relative abundance of amino acids in total protein hydrolysates of Nicotiana.

Constituent	Parental Nicotiana Species: N. tabacum C139	N. tabacum SC58	sylvestris	tom. [sic] fms	otophora	glauca[b]	glutinosa	F_1 Hybrids[a]: tab x syl	tab x tom	tab x oto	tab x gla	tab x glu	L.S.D. .05	L.S.D. .01	CV (%)
	Moles per 1000 units, (Average of 3 replications)														
Tryptophan Deriv.	2.7	2.7	3.7	3.0	2.7	1.8	1.7	3.0	2.3	3.3	2.6	2.0	1.0	1.4	24
Lysine	37.3	38.0	42.3	41.7	46.3	44.2	41.3	41.5	43.2	44.4	43.5	43.6	3.2	4.3	5
Histidine	15.0	14.3	14.0	14.3	16.0	15.5	12.0	15.0	14.6	15.0	14.1	15.0	1.4	1.9	6
Unknown A	4.3	5.0	4.0	4.0	5.3	3.0	3.0	5.0	4.2	5.2	3.8	4.5	0.9	1.2	13
Ammonia	106.0	108.0	111.0	126.0	108.0	102.5	107.0	108.0	120.0	109.0	125.8	107.0	7.0	9.0	4
Arginine	31.7	31.7	33.7	33.3	35.3	31.0	31.3	32.4	30.6	33.2	31.6	30.2	2.5	3.3	5
Unknown B	1.3	1.0	1.0	1.0	0.8	1.0	1.3	1.0	1.0	1.0	1.0	1.0	NS	NS	2
Unknown C	11.0	8.0	3.7	3.0	2.7	1.5	1.7	4.5	5.2	4.0	4.6	5.2	4.0	5.3	54
Unknown D	7.7	6.3	3.3	3.3	2.7	2.3	1.7	5.7	5.2	5.2	4.2	5.3	0.8	1.1	11
Hydroxyproline	21.3	30.7	25.7	19.7	16.7	16.4	14.3	34.5	29.6	36.2	30.9	33.2	11.0	14.8	24
Unknown E	0.7	0.5	0.8	1.0	0.8	0.8	1.0	0.9	0.8	0.8	0.9	0.9	0.4	0.5	26
Aspartic Acid	88.7	88.7	87.3	88.7	90.3	86.4	92.0	87.0	88.8	86.5	91.2	87.4	3.1	4.2	2
Threonine	44.7	43.7	42.7	43.7	45.3	41.8	43.3	43.8	43.0	44.5	42.4	42.2	1.6	2.1	2
Serine	53.7	53.7	51.3	51.3	53.7	49.6	51.3	52.2	50.5	53.2	51.4	50.7	2.1	2.8	2
Glutamic Acid	89.3	86.3	81.0	83.7	85.7	88.0	87.7	86.6	86.2	84.5	84.1	88.4	2.6	3.5	2
Proline	52.7	51.7	50.7	52.7	53.0	51.0	50.0	50.8	51.2	51.8	49.2	51.0	2.1	2.9	3
Glycine	88.3	89.0	99.3	92.3	90.0	92.8	93.7	88.6	88.0	86.8	87.6	89.8	2.1	2.8	1
Alanine	79.0	78.0	78.3	78.3	78.3	82.2	83.0	78.2	77.4	76.4	76.1	78.8	2.7	3.6	2
Cystine	2.0	1.2	6.0	3.0	2.7	2.7	3.7	3.0	1.0	2.2	3.6	2.0	1.7	2.3	38
Valine	66.0	65.0	62.3	63.7	63.3	66.4	67.3	63.3	65.4	62.4	62.8	65.3	2.6	3.5	2
Methionine	12.0	11.0	10.7	11.3	11.3	12.4	12.7	10.7	10.4	11.5	10.3	11.3	1.3	1.8	7
Unknown F	1.0	0.8	1.0	1.0	1.0	1.0	1.0	1.1	1.2	0.8	1.0	1.0	0.3	NS	18
Isoleucine	47.3	46.7	45.7	44.3	45.3	49.3	49.3	46.5	45.6	45.5	44.4	47.2	3.2	4.3	4
Leucine	81.3	79.0	78.0	77.7	80.0	89.6	84.3	79.2	77.5	77.2	74.8	79.5	6.1	8.2	5
Tyrosine	19.0	19.3	21.0	20.0	21.7	21.2	21.0	19.4	19.0	20.8	19.8	19.2	1.7	2.3	5
Phenylalanine	40.7	40.0	43.3	42.3	42.3	48.0	45.3	40.5	40.5	40.8	40.1	41.2	3.8	5.1	5

[a] *Hybrids involving C139 and SC58 have been averaged together, as have also those of the two glaucas.*
[b] *Average of the "yellow" and "purple" varieties.*

Table 3a. Means and statistical significance of certain contrasts: Protein hydrolysates (moles/1000 units).

Constituent	Among Parents	Among Hybrids	Weighted Means of Parents	Weighted Means of Hybrids	(F)	F_1 Hybrids of C139	F_1 Hybrids of SC58	(F)	Among Species in Hybrids	Among tab x spec. in Hybrids
Tryptophan Deriv.	**	*	2.6	2.6	NS	2.8	2.5	NS	**	NS
Lysine	**	NS	40.5	43.3	**	42.7	43.8	NS	NS	NS
Histidine	**	NS	14.6	14.6	NS	14.6	14.7	NS	NS	NS
Unknown A	**	**	4.2	4.4	NS	4.3	4.5	NS	**	*
Ammonia	**	**	108.0	116.0	**	116.0	116.0	NS	**	NS
Arginine	*	NS	32.1	31.6	NS	31.4	31.8	NS	*	NS
Unknown B	NS	NS	1.1	1.0	NS	1.0	1.0	NS	NS	NS
Unknown C	**	NS	5.9	4.7	NS	4.9	4.4	NS	NS	NS
Unknown D	**	**	4.8	5.0	NS	5.3	4.7	**	**	**
Hydroxyproline	NS	NS	22.1	32.6	**	32.3	32.8	NS	NS	NS
Unknown E	NS	*	.7	.9	NS	.9	.9	NS	NS	**
Aspartic Acid	**	**	88.6	88.7	NS	88.2	89.2	NS	**	NS
Threonine	**	**	43.6	43.1	*	43.2	42.9	NS	**	NS
Serine	**	**	52.4	51.6	*	51.6	51.6	NS	**	NS
Glutamic Acid	**	**	86.8	85.6	*	86.2	85.1	NS	**	NS
Proline	*	*	51.8	50.5	**	50.9	50.2	NS	**	NS
Glycine	**	*	91.1	88.1	**	87.8	88.3	NS	**	NS
Alanine	**	NS	79.4	77.1	**	77.4	76.8	NS	*	NS
Cystine	**	**	2.5	2.6	NS	2.3	2.8	NS	**	NS
Valine	**	**	65.2	63.6	**	63.6	63.7	NS	**	*
Methionine	*	NS	11.6	10.7	**	10.9	10.6	NS	*	NS
Unknown F	NS	**	1.0	1.0	NS	.9	1.1	**	**	NS
Isoleucine	**	NS	47.1	45.6	**	45.6	45.6	NS	NS	NS
Leucine	**	NS	81.7	77.2	**	77.0	77.3	NS	NS	NS
Tyrosine	*	NS	20.1	19.7	NS	19.4	19.9	NS	*	NS
Phenylalanine	**	NS	42.6	40.5	**	40.7	40.3	NS	NS	NS

* *Difference significant at P = .05*
** *Difference significant at P = .01*
NS *Nonsignificant difference at P = .05*

detectable in either *N. tabacum* variety but were reliably estimated in each of the other species. Quantitatively, *N. tabacum* was lower than the other species in lysine, histidine, ammonia, arginine, aspartic acid, STAG, glycine, valine, isoleucine, leucine, phenylalanine, and γ-aminobutyric acid; both varieties were higher in taurine, L_4, methionine-S-oxide, proline, α-aminoadipic acid, tyrosine, galactosamine, and L_{11}. No clear patterns of parental differences were evident for cysteic acid, glutamic acid, alanine, or glucosamine.

The free amino acid compositions of the two varieties of *N. tabacum* (**Table 1, Columns 1 and 2**) were generally quite similar. By comparing the differences between their means against the L.S.D., these varieties differed significantly for only four constituents. C139 was higher in methionine-S-oxide and in proline, whereas SC58 contained more taurine and glutamic acid.

(2) *Among F_1 Hybrids.* In general, the range in concentration for most of the free amino acids among the hybrids was somewhat less than the range among the parents. Notable exceptions were the pronounced increases in proline and glucosamine in *N. tabacum* x *N. glutinosa*, and the conspicuous reduction in galactosamine in *N. tabacum* x *N. glauca*. This reduced variability is reflected in the statistics; differences in arginine, valine, isolencine, and leucine among hybrids were nonsignificant (**Table 1a**, Column 2), whereas the species differed significantly in these and all other constituents.

(3) *Parent-Hybrid Relationships.* In **Table 1a**, weighted means[4] of all parents (Column 3) for each constituent are compared with the means of all hybrids (Column 4); the statistical significances of the deviations of the hybrids from the parents are indicated in the adjacent column headed (F) (Column 5). On the average, the F_1 hybrids were significantly higher than the parents in histidine, ammonia, cysteic acid, aspartic acid, STAG, proline, alanine, phenylalanine, galactosamine, and L_{11}, but contained lower concentrations of methionine-S-oxide, glutamic acid, α-aminoadipic acid, valine, isoleucine, leucine, and tyrosine.

Some measure of parental influence on the amino acid compositions of their hybrid progenies can be deduced from **Table 1a** where, in Columns 6 and 7, the means of the C139 offspring are compared with those of SC58. The significantly higher concentrations of methionine-S-oxide and tyrosine as well as the lower level of glutamic acid in the C139 hybrids are in agreement with differences between the tobacco parents *per se*. However, the excess of taurine in SC58 over C139 was not carried over into its hybrids. The two groups of

Table 4. Nitrogen fractions and alkaloid contents of Nicotiana species and interspecific hybrids.

Parent or Hybrid	Total Nitrogen	Protein Nitrogen	Total Alkaloid	Nicotine	Nor-nicotine
	In per cent, (Average of 3 replications)				
N. tabacum (C139)	2.03	1.80	1.89	1.80	.09
N. tabacum (SC58)	2.58	1.65	4.13	3.96	.16
N. sylvestris	2.92	1.74	1.74	.73	.92
N. tomentosiformis	2.81	2.59	.30	.21	.10
N. otophora	3.00	2.25	.19	.16	.03
N. glauca (γ)	3.69	2.34	.94	.42	.55[a]
N. glauca (p)	3.14	2.63	.67	.37	.27[a]
N. glutinosa	4.51	3.81	4.56	1.26	3.02
C139 x N. sylvestris	2.42	1.28	1.78	.72	.97
SC58 x N. sylvestris	2.94	1.97	3.34	1.13	2.01
C139 x N. tom'fms	2.64	2.33	.87	.48	.42
SC58 x N. tom'fms	2.60	1.79	1.23	.56	.61
C139 x N. otophora	2.16	1.24	.85	.29	.58
SC58 x N. otophora	2.45	1.50	1.11	.48	.57
C139 x N. glauca (γ)	3.01	1.74	.81	.35	.42[a]
SC58 x N. glauca (γ)	3.84	2.91	2.47	.69	1.70[b]
C139 x N. glauca (p)	2.85	1.81	.81	.37	.40[b]
SC58 x N. glauca (p)	3.31	1.82	1.93	.86	.97[b]
C139 x N. glutinosa	2.71	1.66	2.98	.99	1.83
SC58 x N. glutinosa	3.98	2.56	6.37	1.70	4.44
LSD .05	.36	.49	.57	.21	.54
.01	.48	.66	.77	.28	.73
Among parents	**	**	**	**	**
Among F_1 hybrids	**	**	**	**	**
Parent means (weighted)	2.82	2.14	2.20	1.70	.47
F_1 means	2.91	1.88	2.06	.72	1.24
Parent vs F_1 mean	NS	**	NS	**	**
C139 F_1 mean	2.63	1.68	1.35	.53	.77
SC58 F_1 mean	3.19	2.09	2.77	.90	1.72
C139 vs SC58 F_1 means	**	**	**	**	**
Among species/hybrids	**	**	**	**	**
Tab. x species/hybrids	**	**	**	**	**
CV (%)	7	14	17	14	32

[a] *Probably mostly anabasine but analyzed as nornicotine.*
[b] *Both nornicotine and anabasine, as nornicotine.*
** *Difference significant at P = .01*
NS *Nonsignificant difference at P = .05*

[4] *Weighting of the parental means is necessary to account for the relative frequency of their involvement in hybrid combinations. As can be seen from the listing of the individual hybrids in Table 4, each variety of N. tabacum occurs in six hybrids whereas another species, N. sylvestris for example, is involved in only two. Thus, each N. tabacum variety is included three times in the weighted mean.*

Table 5. Percentage heterosis[a] displayed by F, interspecific hybrids.

Constituent	Free Amino Acids in F_1, tab x					In Hydrolysates of F_1, tab x				
	syl.	tom'f.	oto.	gla.	glu.	syl.	tom'f.	oto.	gla.	glu.
Tryptophan Deriv.						— 8	— 16	— 2	12	— 31
Lysine	— 26	42	— 39	47	5	6	20	6	21	19
Histidine	— 1	232	58	20	11	10	11	— 4	6	21
Ammonia	— 12	20	37	70	43	2	6	— 7	33	— 31
Arginine	2	21	73	192	171	1	5	1	15	3
L_{11} (Cysteic Acid)	0	1	7	35	22					
Unknown B						— 33	— 25	— 23	— 19	— 50
Unknown C						— 38	— 22	— 48	— 9	— 7
Taurine, (L_8)	38	44	31	— 54	80					
Unknown D						5	— 1	— 2	— 3	10
Hydroxyproline						58	56	84	73	82
Unknown E						4	17	9	— 13	— 28
Methionine-S-oxide	40	— 21	— 10	— 71	— 32					
Aspartic Acid	29	70	— 12	44	— 23	2	9	— 3	19	4
STAG	— 25	59	12	91	— 28					
Threonine						3	7	— 2	13	4
Serine						3	4	— 2	14	5
Glutamic Acid	— 6	44	3	— 32	— 14	7	9	— 3	9	9
Proline	93	57	27	— 57	319	3	7	— 2	9	7
α-Aminoadipic Acid	18	— 20	18	— 83	— 24					
Glycine	— 25	25	3	27	— 12	— 3	6	— 3	10	6
Alanine	82	39	15	35	31	4	7	— 3	8	5
Cystine						— 21	— 53	15	95	— 15
Valine	— 39	39	32	29	— 72	3	11	— 3	8	6
Methionine						4	— 10	17	— 19	— 10
Unknown F						52	— 12	— 28	— 13	— 14
Isoleucine	— 30	38	— 9	30	— 75	5	8	— 3	4	5
Leucine	— 42	35	11	52	— 86	5	8	— 3	2	4
Tyrosine	7	— 4	— 2	— 69	— 29	0	9	3	13	0
Phenylalanine	— 10	89	71	81	6	— 1	7	— 2	3	4
Glucosamine	— 3	68	48	— 14	453					
Galactosamine	114	99	91	— 17	336					
L_{11} (Unknown)	52	32	46	— 37	119					
γ-Aminobutyric Acid	9	20	— 5	— 13	21					

[a] *Percentage deviation of F_1 hybrid above average of parents in cross. (Negative value indicates F_1 hybrid below average of parents).*

hybrids differed significantly in nine additional constituents for which the differences between the two varieties were not significant. For six of these—lysine, STAG, α-aminoadipic acid, phenylalanine, glucosamine, γ-aminobutyric acid—the direction of the differences between the hybrid families corresponded to that of the parents, but for aspartic acid, glycine, and L_{11}, the relationship was reversed.

With regard to the influence of the other (species) parent, i.e., (C139 x *N. sylvestris* plus SC58 x *N. sylvestris*) versus (C139 x *N. otophora* plus SC38 x *N. otophora*), etc., the average specific differences (**Table 1a**, Column 9) were significant for the same constituents for which hybrids differed generally (Column 2).

The last column in **Table 1a** is concerned with parental interactions as expressed in their F_1 hybrids. The eight interactions indicated as significant are detailed in **Table 2**. In this table, the data for each of the eight constituents are presented as the average difference between the hybrids of SC58 x any species and of C139 x same species. Without exception, the C139 hybrids exceeded the SC58 crosses in methionine-S-oxide irrespective of the other parent; the magnitude of these differences ranged from a low of 62 μg/gm in hybrids involving *N. glauca* to a high of 568 μg/gm in crosses with *N. sylvestris*. For the other seven constituents, the differences fluctuated from minns to plus depending upon the particular species used as the other parent. Among *N. tomentosiformis* hybrids, those involving C139 exceeded those of SC58 in seven of the eight constituents (the exception being glucosamine) while, in the *N. glutinosa* group, the SC58 half were higher in every constituent except methionine-S-oxide.

Protein Hydrolysates. Concentrations of amino acids, expressed as "moles per 1000 units," in total protein hydrolysates are presented in **Table 3** and the statistical tests in **Table 3a.** The inclusion of ammonia does not imply that ammonia *per se* is a constituent of *Nicotiana* proteins, but it is always present in the hydrolysis mixture. Similarly, while Unknowns A, B, C, D, E, and F were present in every hydrolysate, they most likely are artifacts.

Relative to free amino acid extracts, the analytical precision on hydrolysate mixtures is far superior as evidenced by the very much smaller coefficients of variation (last column, **Table 3** versus **Table 1**).

(1) *Among Parental Species.* Comparing their compositional differences against the L.S.D., the two varieties of *N. tabacum* differed in only two constituents; C139 was higher than SC58 in unknown D and glutamic acid. In broader aspect, this group of parental species differed significantly in every true amino acid. Only unknowns B, E, F, and hydroxyproline were not different among these species (**Table 3a,** Column 1).

(2) *Among Hybrids.* The lesser variability among hybrids is evidenced by the fewer constituents that tested significant (**Table 3a,** Column 2 versus Column 1). In accord with the species, hybrids also differed significantly in ammonia, unknowns A and D, aspartic acid, threonine, serine, glutamic acid, proline, glycine, cystine, and valine; neither hybrids nor parents differed in unknown B or hydroxyproline. At variance with species behavior, hybrids did not differ in lysine, histidine, arginine, unknown C, alanine, methionine, isoleucine, leucine, tyrosine, and phenylalanine—constituents that differed significantly among the parents. On the other hand, unknowns E and F differed significantly in the hybrids but not in the species.

(3) *Parent-Hybrid Relationships.* From the weighted parent[4] and hybrid means in **Table 3a,** the hybrid hydrolysate contained higher concentrations of lysine, ammonia, and hydroxyproline than did the parents, but yielded lower proportions of threonine, serine, glutamic acid, proline, glycine, alanine, valine, methionine, isoleucine, leucine, and phenylalanine.

Hydrolysates of the hybrids of C139 contained more of unknown D but lower concentrations of unknown F than the SC58 hybrids (**Table 3a,** Columns 6, 7, and 8); the former difference is in agreement with the contrast between the parental varieties. SC58 and C139 *per se* did not differ in unknown F.

The two varieties SC58 and C139 interacted in crosses with the other species only for unknowns A, D, and E, and valine (**Table 3a,** last column).

Nitrogen Fractions and Alkaloids. Average concentrations of total nitrogen and protein nitrogen as determined by a modified Kjeldahl procedure (7) and of total alkaloid, nicotine and nornicotine by the Cundiff and Markunas method (3) for each experimental entry are given in **Table 4.**

All constituents varied widely both among species and among hybrids. SC58 was significantly higher than C139 in total nitrogen, total alkaloid, and nicotine.

After weighting,[4] the hybrids averaged lower in protein nitrogen than the parents, but were not different in total nitrogen. Hybrids and parents were quite similar in total alkaloid contents, but the hybrids were lower in nicotine and higher in nornicotine. Hybrids having SC58 as the common parent were higher in all constituents than were the C139 hybrids; these differences, with the exception of protein nitrogen, are in the same order as the comparison between the varieties *per se.*

Hybrid combinations involving either SC58 or C139 with another species accumulated nitrogenous compounds quite differently. The SC58 half of the *N. glutinosa* crosses were significantly higher than the C139 group for each constituent. Yet in other families, e.g., *N. tomentosiformis* or *N. otophora,* comparable contrasts were not large. This erratic behavior accounts for the significant interactions, "*N. tabacum* x species/hybrids" (**Table 4,** bottom line).

DISCUSSION

Of the 20 entries with which this experiment was concerned, only the two flue-cured representatives of *N. tabacum,* SC58 and C139, have commercial value and, consequently, are the only ones that man has bothered to learn how to culture and manage. All of the other species grow wild except as they may be brought into a genetic nursery to serve as a pollen or seed parent and, under such circumstances, their vegetative well-being is largely ignored. The interspecific hybrids, even though some require considerable effort to make, exist only to satisfy man's experimental curiosity.

It is recognized therefore that the management aspects of this experiment were unintentionally, but nonetheless unavoidably, biased in favor of SC58 and C139. Some of the cultural "mismanagements" that likely would have influenced the analytical findings include:

Nutrition. Over the years while tobacco (*N. tabacum*) has been produced commercially, man has studied and learned the nutritional requirements of the flue-cured plant. Fertilization of this experiment conformed to the reccommendations for flue-cured production and thus was near optimal for SC58 and C139. The other entries may have quite different requirements. Considering size alone, a small plant—*N. glutinosa,* for example—would have been grossly overfertilized; *N. glutinosa* was significantly higher in total nitrogen than all of the other entries. On the other end of the scale, certain of the hybrids exhibited extreme heterosis for growth (5) and may have been undernourished; one of these, C139 x *N. otophora,* was lowest in total nitrogen.

Topping. In practical tobacco culture, the "crop" is made up of the cured leaves and so, in order to avoid the nutrient (yield) drain required to form the seeds, topping is a recommended practice. Insofar as possible, the entries in this experiment were topped at the proper stage of development. However, floral initiation, usually measured as "days-to-flower," is a genetic trait and differed widely among these experimental materials (5). Even at the end of the harvest period, *N. otophora, N. tomentosiformis,* and *N. glauca* still had not flowered. Thus the analytical samples of these entries were from non-topped plants and, as a consequence, *N. otophora,* and *N. tomentosiformis* were lower both in total alkaloids and in degree of conversion of nicotine into nornicotine than is normally found in topped samples of these species (unpublished). By contrast, *N. glutinosa* had flowered and was topped on the 18th day following transplanting; it had accumulated extremely high concentrations of total alkaloid.

Harvesting and Curing. Although optimum ripeness of a tobacco leaf cannot be precisely defined other than that it is on the senescent side of physiological maturity, still experienced growers have no trouble in recognizing ripeness in leaves of familiar tobacco varieties. SC58 and C139 were harvested at or near optimal ripeness. As best ripeness could be judged in the other unfamiliar species and hybrids, they too were harvested when ripe but, most likely, these samples also included under-ripe or even immature leaves.

Flue-curing is still more of an art than a science, yet an experienced operator can and does appropriately adjust the curing schedule to suit the degree of ripeness and node position of the particular harvest. To some degree, under-ripeness can be overcome by extending the yellowing period. Both the degree of ripeness at harvest and the duration of the yellowing period rather markedly influence the chemical composition of the cured product (2). The samples in this experiment were all cured together but on a schedule adapted to the ripe SC58 and C139 leaves.

Analytically, free amino acid extracts of green leaves, particularly immature leaves but also ripe ones, contain only traces or none of the ninhydrin-positive compounds other than the true amino acids. These other constituents, of which taurine, α-aminoadipic acid, galactosamine, and L_{12} are examples, are formed mostly during curing; moreover their levels of accumulation are directly related to maturity (unpublished data). Because these compounds do react with ninhydrin, they are presumed to be metabolic derivatives of amino acids. Curiously, it was in these metabolic products that the two *N. tabacum* varieties were most conspicuously higher than all of the other entries, both species and hybrids (**Table 1**). Both of the varieties were also significantly lower in certain amino acids; it is quite conceivable that some of these amino acids were the parent substances from which the metabolites were derived. Therefore, it seems reasonable that some of these quantitative differences are at least partially attributable to the relative immaturities of these unfamiliar materials.

In addition to the cultural confounding that has just been described, the experiment was also confounded genetically. Three levels of polyploidy are represented among the 20 entries: the two varieties of *N. tabacum* are amphidiploids; all of the other parental species are diploids; the hybrids are all triploids. Matzinger and Wernsman (5) were unable clearly to associate differences in agronomic performances among these 20 lines with degree of polyploidy. Any effects of polyploidy are even less discernible in the present data.

Among a group of genetically divergent materials such as these, one might have presupposed that any differences in the constituents "caught" in the free amino acids pools would be of a quantitative nature only and, further, that such differences would be more responsive to environmental influences than to genetic requirements. And indeed, the effects of some external variables on the levels of certain free constituents have just been pointed out.

A further extension of this rationale might suppose that the true genetic effects, as expressed in hybrid combinations, would be evidenced in the protein hydrolysates. From even a cursory glance at the hydrolysates

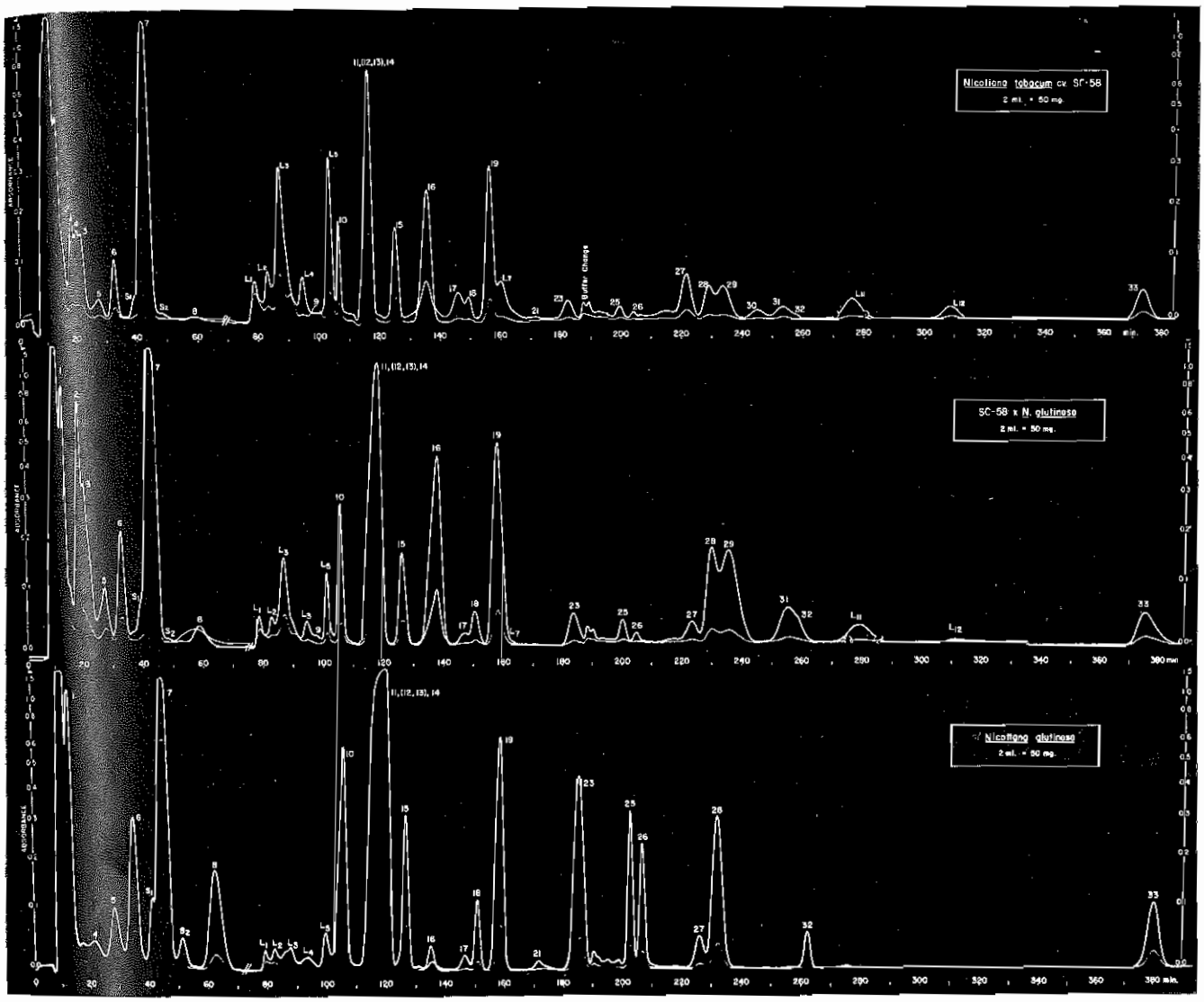

Figure 1—Analytical chromatograms of the free amino acids in *Nicotiana* extracts: *(Upper) N. tabacum* cv SC58; *(Middle)* F_1 hybrid, SC58 x *N. glutinosa*; *(Bottom) N. glutinosa*. Peak identifications: (1) acidic and neutral compounds; (2) glucosamine; (3) galactosamine; (4) tryptophan; (5) lysine; (6) histidine; (S_1) unknown; (7) ammonia; (S_2) unknown; (8) arginine; (L_1, L_2, L_3, L_4) unresolved mixture of acidic compounds probably including peptides and amino acid-sugar complexes; (L_5) may include cysteic acid; (L_6) possibly taurine; (9) hydroxyproline; (L_8) methionine-S-oxide; (10) aspartic acid; (11, 12, 13, 14)—STAG—unresolved mixture of serine (14), threonine (11), asparagine (12), and glutamine (13), computed as an equimolar mixture; (15) glutamic acid; (16) proline; (17) α-aminoadipic acid; (18) glycine, (19) alanine; (L_7) unknown; (21) cystine; (23) valine; (25) isoleucine; (26) leucine; (27) tyrosine; (28) phenylalanine; (29) glucosamine; (30) homocystine; (31) galactosamine; (32) β-alanine; (L_{11}) unknown; (L_{12}) unknown; (33) γ-aminobutyric acid.

data (**Table 3**), one is immediately impressed by the utter absence of qualitative differences—a unique presence of one constituent or a conspicuous absence of another. Every constituent was present in each hydrolysate. Statistically significant quantitative differences were plentiful (**Table 3a**), but the magnitudes of these differences were generally too small to be exciting biologically. Included in the statistical analyses were certain specific comparisons that would be meaningful genetically. Prominent among the relatively few constituents that tested significant in these comparisons were the unknowns A, D, and F; these compounds are believed to be artifacts of the hydrolysis and not components of tobacco proteins. A possible exception is hydroxyproline. It is not definitely known whether hydroxyproline is or is not a component of tobacco proteins. It was present in every hydrolysate in abundances ranging from 14.3 to 36.2 moles per 1000 units (**Table 3**); moreover hydroxyproline was found among the free amino acids of some entries (**Table 1**). On the other hand, its high analytical variability (C.V. = 24%) favors the artifact argument.

It should be emphasized that these hydrolysate data are for *total* protein hydrolysates. It is not inconceivable that particular proteins could be grossly different among genetic lines without being detected in the adulterated mixtures.

The free amino acid extracts of all entries contained at least trace amounts of all of the true α-amino acids. But voids in the data—qualitative differences—are clearly evident in **Table 1.** With the exception of cystine that was not detected in either variety of *N. tabacum* nor in the *N. tabacum* x *N. sylvestris*, all of the other missing constituents are metabolic derivatives of amino acids, indicating biochemical aberrations (relative to *N. tabacum* as the standard). Moreover, the differential patterns among the hybrids suggest that the responsible biochemical mechanisms are amenable to genetic manipulation.

The sharp qualitative contrasts are those between *N. tabacum* on the one hand as against the other species as a group; a compound was present in both *N. tabacum* varieties but absent in the other species or *vice versa*. In most instances, the closely related hybrids—those between *N. tabacum* with each of the three putative

progenitor species—respond like *N. tabacum*, while the distant hybrids behave like their wild parent. Specific examples follow. The free amino acid extracts of both varieties of *N. tabacum* contained hydroxyproline, the species extracts did not. Hydroxyproline was present in extracts of *N. tabacum* x *N. sylvestris*, of *N. tabacum* x *N. tomentosiformis* and of *N. tabacum* x *N. otophora* (progenitors), but was not found in *N. tabacum* x *N. glauca* nor in *N. tabacum* x *N. glutinosa*. In the opposite direction, β-alanine which is a derivative of aspartic acid was present in all of the wild species and in most of the hybrids; this metabolite was not detected in the extracts of either variety of *N. tabacum* nor in *N. tabacum* x *N. sylvestris*.

The *N. glutinosa* family displayed more examples of qualitative differences than any other. For this reason, the free amino acids chromatograms of *N. tabacum* (SC58), of *N. glutinosa*, and of SC58 x *N. glutinosa* have been reproduced in **Figure 1.** The chromatogram of *N. glutinosa* shows no evidence of L_7, glucosamine, homocystine, galactosamine, L_{11}, or L_{12}. Peaks of these compounds are identified on the chromatogram of the *N. tabacum* parent. All of these except L_7 and homocystine were also present in the hybrid; concentrations of glucosamine and galactosamine exceeded those of SC58 (heterosis).

Deviation of a F_1 hybrid from the mean of its parents is evidence of heterosis; values higher than the average of the parents are called positive heterosis, lower values are negative. Matzinger and Wernsman (5) found heterosis values for most agronomic characters to be highest in crosses between *N. tabacum* and a putative progenitor in the order of *N. otophora*, *N. tomentosiformis*, and *N. sylvestris* and lowest in the more distantly related hybrids involving *N. glauca* and *N. glutinosa*. Heterosis values for both the free amino acids and protein-bound amino acids are given in **Table 5.** While specific examples showing a similar distinction between near and distant relatives of *N. tabacum* might be found in these data, the pattern is anything but discrete. Positive and negative values are about equally abundant and nearly randomly distributed. Several values exceed 100%.

SUMMARY

Flue-cured samples of 20 diverse genetic lines including six species—*N. tabacum* (flue-cured varieties SC58 and C139), *N. sylvestris*, *N. tomentosiformis*, *N. otophora*, *N. glauca* (yellow and purple flowered accessions), *N. glutinosa*—and 12 F_1 hybrids of each tobacco variety crossed with each of the other species were analyzed for free amino acids and for amino acids liberated from their total proteins by hydrolysis. Hydrolysates differed significantly among entries for many components but the magnitudes of these differences were generally too small to suggest biological importance. Large quantitative and discrete qualitative differences were found in the free amino acid extracts of these divergent materials. For missing constituents, hybrids involving progenitor species most often resembled the *N. tabacum* parent, whereas the more distantly related hybrids emulated their wild parents. These data suggest that the responsible biochemical mechanisms might be amenable to genetic manipulation. No attempt was made to correlate these results with tobacco quality.

LITERATURE CITED

1. Burde, R. de la, E. H. Poindexter, Jr., and J. P. Bell. "The distribution of amino acids in tobaccos from different stalk positions." *Tobacco Science*, 9: 26-32, 1965.

2. Carr, J. M., W. G. Woltz, J. A. Weybrew, and R. J. Monroe. "Curing studies (1965): Ripeness x rate of curing." 21st Tobacco Worker's Conference, Williamsburg, Va., January 17-20, 1967. (Unpublished)

3. Cundiff, R. H., and P. C. Markunas. "Determination of nicotine, nor-nicotine and total alkaloids in tobacco." *Anal. Chem.*, 27: 1650-1653, 1955.

4. Hamilton, P. B. "Gasometric determination of glutamine amino acid carboxyl nitrogen in plasma and tissue filtrates by the ninhydrin-carbon dioxide method." *J. Biol. Chem.*, 158: 375-395, 1945.

5. Matzinger, D. F., and E. A. Wernsman. "Genetic diversity and heterosis in *Nicotiana*. I. Interspecific crosses." *Der Zuchter*, 37: 188-191, 1967.

6. McKenzie, H. A., and H. S. Wallace. "The Kjeldahl determination of nitrogen: A critical study of digestion conditions—temperature, catalyst, and oxidizing agent." *Austr. J. Chem.*, 7: 55-60, 1954.

7. Official Methods of Analysis of the A.O.A.C. 8th Ed. Association of Official Agricultural Chemists, Washington, 1955. Sec. 2-24.

8. Spackman, D. H. In Instruction Manual AIM-2, Spinco Division, Beckman Instruments Inc., Palo Alto, California, 1962. Part 7, Page 2.

9. Weybrew, J. A., and T. J. Mann. "Comparative composition of *Nicotiana* species and their F_1 hybrids with *N. tabacum*." *Tobacco Science*, 7: 28-36, 1963.

10. Zacharius, R. M., and E. A. Talley. "Elution behavior of naturally occurring ninhydrin-positive compounds during ion-exchange chromatography." *Anal. Chem.*, 34: 1551-1556, 1962.