observed under very mild conditions show the characteristics expected from free ellagic acid, it is considered proved that the precipitate is predominantly free ellagic acid rather than an ellagitannin, free hexahydroxydiphenic acid, or other possible precursors of ellagic acid.

Ellagic acid has a very low solubility in aqueous solutions and is readily removed from solution or suspension by most fining, filtration, or adsorptive treatments. It therefore appears that the ellagic acid precipitate results from the slow generation of ellagic acid. In preliminary experiments with canned loganberries, free ellagic acid was not found. It appears most probable that the source of the ellagic acid is an ellagitannin present in the loganberries which is relatively soluble in wine and is produced or extracted in variable amounts to account for the different degree of instability of different lots of loganberry wine. The slow rate of formation of the crystalline precipitate presumably reflects the slow hydrolysis of the more soluble ellagitannin and perhaps delayed lactonization of the resultant hexahydroxydiphenic acid. It is possible, of course, that enzymes present in the berry or even "tannase" produced in an occasional moldy berry are involved and synthesis from simpler substances such as gallates could occur.

These considerations are strengthened by the fact that improved, but not always completely stable, clarity of the unstable loganberry wine has been achieved by heating or treatment with gelatin or activated carbon. It is probably significant that although we were unable to find a report of the presence of ellagates in loganberries, ellagic acid has been reported from raspberries (*11*). The loganberry is reported to be a cross between European red raspberries and California blackberries. A precipitate very similar to that described here has been noted in a commercial red raspberry wine (*1*). We have been able to locate no previous report of wine turbidity or sediment produced by ellagic acid, which is interesting in view of reports of its presence in grapes and grape wines (*5*, *6*).

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AMINO ACIDS OF HYBRIDS

Alien Genome Combinations and Influence on Amino Acid Composition of Cereal Protein Fractions

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Comparisons were made of the amino acid composition of comparable protein fractions of rye (*Secale cereale–RR*), durum (*Triticum durum*–AABB), *Triticale*, the synthetic species comprised of AABBRR, and *Tritipyron*, a synthetic species composed of genomes AABBEE. Proteins of a common wheat (*Triticum vulgare*) which has the genomic constitution of AABBDD were also investigated. In most cases, the amino acid composition of the "whole" proteins of *Triticale* was intermediate between that of the two parents, rye (RR) and durum (AABB). Differences in amino acid composition of protein fractions of *Triticale*, however, reflected a dominant effect of either parental genome (RR or AABB) or were intermediate between the two parents. A decrease in the individual amino acid of one protein fraction of *Triticale* was, in general, compensated for by an increase in the same amino acid in another fraction. In the case of the synthetic species *Tritipyron* (AABBEE) and common wheat (AABBDD), data for the parental genomes EE and DD are not available and any differences in the observed amino acid patterns from that of durum (AABB) are tentatively ascribed to the influence of the EE and DD genomes.

STARCH-gel electrophoresis studies of proteins (*10*) using aluminum lactate–urea buffer (*4*, *9*) indicated some variable effects attributable to consequences of alien genome combinations. In the investigations on some protein fractions derived from a synthetic rye-wheat hybrid, *Triticale* (*10*), it was found that although a general additive effect pervaded, a number of what were believed to be new or different hybrid substances were elaborated. The general additive effect of alien genome combinations had been noted (*6*, *7*) early in the development of new synthetic cereal species. Some differences were later detected by immunologic techniques (*1*) and by starch-gel electrophoresis (*10*) due

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to its relatively great resolving power of cereal proteins. Johnson and Hall (*3*) have shown that the gel electrophoresis protein spectra of the A and B genomes were different and some divergence had occurred within the AABB species in that the A genome of *T. dicoccum* (AABB) was only partially homologous with the *T. monococcum* genome (AA). They further showed that different degrees of affinities existed among the A, B, and D genomes and it appeared that the protein spectrum was proportional to the number of genomes that had been contributed to a species. It is not improbable that a genomic interaction could result in the formation of hybrid substances. Experiments with interspecific hybrids of Lotus (*2*) have indicated the formation of a new or different polyphenolic compound, while hybrid enzymes have been described (*8*) for maize.

A major breakthough in the study of cereal proteins, which for the most part might be considered as storage proteins, was the discovery of the extensive resolving power of starch-gel electrophoresis using the aluminum lactate–urea buffer (*4, 9*). This procedure has been used extensively to study a number of related aspects of cereal and other proteins. Since some differences had been noted in the protein pattern of the synthetic species, *Triticale* and *Tritipyron*, when compared to the corresponding patterns of the parental species (*10*), the amino acid composition of the protein fractions was determined. It does not seem improbable that alterations in electrophoretic mobilities could be further observed as differences in the amino acid spectra of the proteins.

Experimental Methods

The procedure whereby the synthetic species or alien genome combinants are elaborated has been referred to (*10*). The stocks of seed were produced under similar environmental conditions. The procedures used in the extraction of the protein fractions (salt-soluble, acid-soluble, alcohol-soluble, and water-soluble) from the seed (5- to 10-pound lots) have also been described (*10*). The freeze-dried protein samples were stored in a deep-freeze until use.

Hydrolysis of Proteins. Suitable quantities (35 to 50 mg.) of proteins were hydrolyzed with 6*N* hydrochloric acid in sealed tubes. The hydrolyzates were filtered and evaporated to dryness.

Amino Acid Analyses. The complete amino acid composition of each hydrolyzate was determined using a Technicon amino acid analyzer and the amount of each amino acid expressed as mole per cent. Each run required about 18 hours. The results of repeated runs for each of the protein fractions of the four species appear in Table I, while Table II contains the amino acid composition of crude or unfractionated protein. Table III compares the amino acid composition of protein fractions within each species. Differences of less than 3.0 mole % were considered non-significant.

Table I. Amino Acid Composition of Protein Fractions

(Mole per cent)

Amino Acid	Species (RR) Rye	(AABB) Durum	(AABBRR) Triticale	(AABBEE) Tritipyron	(AABBDD) Kharkov
Acid-Soluble Protein Fraction					
Asp.	2.33	3.19	3.75[a]	3.11	2.49[b]
Thre-Ser	7.70	9.19	9.77[a]	8.11[b]	8.93
Glut	33.67	30.47	32.45	33.27[b]	31.75[b]
Prol	17.15	14.40	14.49	14.29	16.91[b]
Gly	7.28	6.05	8.72[a]	6.23	6.85[b]
Alan	3.29	4.11	4.81[a]	3.68[b]	3.63[b]
Val	4.47	4.75	5.25[a]	4.41	4.35[b]
Cyst	1.83	2.28	1.93	2.21	1.83
Meth	1.00	1.41	1.40	1.39	1.22
Isol	2.95	4.20	4.12	3.69[b]	3.69[b]
Leuc	5.27	6.87	7.03[a]	6.41	6.34[b]
Tyro	3.79	2.64	3.11	2.81	2.63
Ph Al	4.33	4.19	3.91[c]	3.99	3.93
Lys	1.37	1.57	2.03[a]	1.55	1.58
Hist	1.29	1.97	1.95	1.71	1.74
Arg	2.03	2.96	2.33	2.69	1.77[b]
Salt-Soluble Protein Fraction					
Asp	3.89	5.15	4.39	4.51[b]	5.39
Thre-Ser	9.31	8.71	8.83	9.57[b]	9.82[b]
Glut	27.95	22.36	28.79[a]	28.53[b]	23.07
Prol	15.55	11.02	15.03	11.79[b]	11.07
Gly	5.43	7.25	5.77	5.56[b]	6.99
Alan	4.59	5.71	4.75	4.99[b]	6.43[b]
Val	5.23	6.08	5.30	5.41[b]	6.10
Cyst	1.93	4.03	3.18	4.55[b]	3.71
Meth	0.89	1.98	1.43	2.13	1.71
Isol	4.38	4.01	4.12	3.47[b]	3.90
Leuc	6.23	6.95	6.43	6.28[b]	7.14
Tyro	1.41	2.83	1.87	2.53	2.42
Ph Al	4.79	3.24	3.07[c]	3.13	3.49
Lys	2.07	2.59	2.03	1.91[b]	2.64
Hist	2.05	2.43	2.09	1.76[b]	2.23
Arg	4.39	5.09	2.86[c]	3.79[a]	3.80[b]
Alcohol-Soluble Protein Fraction					
Asp.	1.91	2.73	2.42	2.29[b]	2.24[b]
Thre-Ser	5.93	5.81	5.64	10.97[b]	6.20[b]
Glut	41.85	39.21	39.81	23.84[b]	42.78[b]
Prol	20.14	15.07	16.83	15.63[b]	16.27[b]
Gly	3.33	3.51	3.04[c]	4.41[b]	2.63[b]
Alan	2.48	3.18	3.09	3.36	2.61[b]
Val	4.25	4.35	4.64[a]	6.52[b]	3.40[b]
Cyst	1.85	2.45	2.05	2.68	2.01[b]
Meth	1.02	1.37	1.29	1.83[b]	1.15
Isol	3.57	4.43	4.69[a]	6.38[b]	4.05
Leuc	5.37	6.70	6.29	7.83[b]	6.21[b]
Tyro	1.55	2.31	1.83	2.91[b]	1.94[b]
Ph Al	5.67	4.33	4.25	5.25[b]	4.71
Lys	0.34	0.79	0.63	1.31[b]	0.54
Hist	4.42	1.73	1.58	2.10	1.60
Arg	0.26	1.93	1.79	2.80[b]	1.62
Water-Soluble Protein Fraction					
Asp.	8.75	6.84	6.25[c]	7.36[b]	10.03[b]
Thre-Ser	8.88	11.81	10.93	12.71[b]	9.18[b]
Glut	25.64	16.89	17.75	15.65[b]	20.46[b]
Prol	13.91	8.76	15.29[a]	13.23[b]	9.00
Gly	5.73	7.84	6.31	6.79[b]	7.63
Alan	5.97	7.47	5.59[c]	6.90[b]	8.18[b]
Val	4.73	7.02	6.37	7.45[b]	5.76[b]
Cyst	2.30	3.73	2.83	4.00	4.17[b]
Meth	1.37	1.66	1.47	1.62	1.50
Isol	3.45	4.21	4.81[a]	4.81[b]	3.32[b]
Leuc	5.58	7.23	6.37	6.95	6.33[b]
Tyro	1.60	2.48	1.91	2.18[b]	2.28
Ph Al	3.89	2.66	3.59	2.87	2.89
Lys	2.54	3.68	2.91	3.09[b]	3.15[b]
Hist	1.71	2.47	2.25	1.95[b]	1.71[b]
Arg	3.95	5.19	5.27	2.37[b]	4.33[b]

[a] Higher than in either parent.
[b] Significant deviation from AABB genome.
[c] Lower than in either parent.

Discussion of Results

Starch-gel electrophoresis of proteins from the parent cereal species and the

genome combinant derived from them indicated that the genomes did not necessarily show their effect in a strictly additive manner in the new cellular environment (*10*). Examination of the data presented in Table I concerned with the amino acid composition of the acid-soluble protein also shows the nonadditive effect in some instances. The protein fraction of *Triticale* showed a significantly higher frequency of aspartic acid, threonine-serine, glycine, alanine, valine-leucine, and lysine than either of the two parents and a significantly lower incidence of phenylalanine. The values for proline, methionine, isoleucine, and histidine are a reflection of the durum genome, while the rest of the values are intermediate or of a "hybrid" level. This dilution effect and the apparent direct reflection of one of the parental genomes were not unexpected; however, the enhancement and depression of a few amino acids would not necessarily be expected and are of considerable interest.

In the case of the synthetic species *Tritipyron*, the influence of the AABB genome may be seen in a number of instances. Where the relative amounts of amino acids are significantly higher or lower than in durum (AABB), this may be ascribed to the influence of the EE genome (*Agropyron elongatum*). Since the spectrum for the EE genome is not known, it is not possible in this case to observe exceptions, as in the case of *Triticale* where the spectra of both parental genomes were determined individually. Similarly, deviations noted in common wheat (AABBDD) may be ascribed to the effect of the DD genome and to evolutionary divergence from the parent AABB genome.

The amino acid spectrum of the salt-soluble fraction *Triticale* (Table I) contained a greater incidence of cases in which the values were intermediate. The levels of proline, glycine, valine, histidine, and lysine were comparable to those found in rye (RR), while threonine-serine level found its counterpart in the durum genome. Glutamic acid in *Triticale* was higher than in either parent genome, while the arginine and phenylalanine levels were lower than in either of the two parents. In the *Tritipyron* species, the levels of 13 amino acids deviated considerably from the levels in the AABB genome and this may be due to the EE genome, while the frequency of deviation in the AABBDD genome was much lower (three deviations).

Examination of the amino acid spectrum of the alcohol-soluble protein fraction of *Triticale* again demonstrated a pattern which was seen in the previously discussed protein fractions, with some exceptions. In only two cases was a significant increase evident in the level for any one amino acid (valine and isoleucine) and in only one instance was the level significantly lower (glycine) than that found for the parent genome with the corresponding lower frequency. The lysine, histidine, and arginine content of the rye protein fraction is strikingly lower than that of durum. In comparison, the values of these three amino acids for the *Triticale* protein fraction are considerably higher than in the rye (RR) genome and appear to reflect the more dominant influence of the durum (AABB) genome. In the *Tritipyron* (AABBEE)

Table II. Amino Acid Composition of Unfractionated Protein from Flour Samples

(Mole per cent)

	Species				
Amino Acid	*(RR) Rye*	*(AABB) Durum*	*(AABBRR) Triticale*	*(AABBEE) Tritipyron*	*(AABBDD) Kharkov*
Asp	5.92	4.63	5.11	4.71	4.31
Thre-Ser	9.23	9.51	9.25	10.05[a]	9.30
Glut	23.90	28.81	24.81	26.50[a]	26.89[a]
Prol	15.68	12.95	14.97	14.49[a]	14.59[a]
Gly	6.49	6.10	6.27	6.05	6.38
Alan	5.87	5.09	5.35	5.07	4.93
Val	6.11	5.65	5.97	5.82	4.71[a]
Cyst	1.17	1.41	1.39	1.71[a]	1.45
Meth	1.25	1.35	1.37	1.50	1.37
Isol	4.55	4.43	4.89[b]	4.66	4.67
Leuc	6.85	7.21	7.54[b]	7.29	7.34
Tyro	0.67	1.32	1.18	1.26	1.29
Ph Al	4.55	4.15	4.44	4.58[a]	4.53[a]
Lys	2.77	2.19	2.36	2.35	2.15
Hist	1.95	2.08	2.01	2.27	2.05
Arg	2.97	3.07	3.09	1.71[a]	3.05

[a] Significant deviation from AABB genome.
[b] Higher than in either parent.

Table III. Comparison of Amino Acid

Amino Acid	*Acid-Soluble*	*Salt-Soluble*	*Alcohol-Soluble*	*Water-Soluble*
	Protein Fractions from Rye (RR)			
Asp	2.33	3.89	1.91	8.75
Thre-Ser	7.70	9.31	5.93	8.88
Glut	33.67	27.95	41.85	25.64
Prol	17.15	15.55	20.14	13.91
Gly	7.28	5.43	3.33	5.73
Alan	3.29	4.59	2.48	5.97
Val	4.47	5.23	4.25	4.73
Cyst	1.83	1.93	1.85	2.30
Meth	1.00	0.89	1.02	1.37
Isol	2.95	4.38	3.57	3.45
Leuc	5.27	6.23	5.37	5.58
Tyro	3.79	1.41	1.55	1.60
Ph Al	4.33	4.79	5.67	3.89
Lys	1.37	2.07	0.34	2.54
Hist	1.29	2.05	0.42	1.71
Arg	2.03	4.39	0.26	3.95
	Protein Fractions from Durum (AABB)			
Asp	3.19	5.15	2.73	6.84
Thre-Ser	9.19	8.71	5.81	11.81
Glut	30.47	22.36	39.21	16.89
Prol	14.40	11.02	15.07	8.76
Gly	6.05	7.25	3.51	7.84
Alan	4.11	5.71	3.18	7.47
Val	4.75	6.08	4.35	7.02
Cyst	2.28	4.03	2.45	3.73
Meth	1.41	1.98	1.37	1.66
Isol	4.20	4.01	4.43	4.21
Leuc	6.87	6.95	6.70	7.23
Tyro	2.64	2.83	2.31	2.48
Ph Al	4.19	3.24	4.33	2.66
Lys	1.57	2.59	0.79	3.68
Hist	1.97	2.43	1.73	2.47
Arg	2.96	5.09	1.93	5.19
	Protein Fractions from Triticale (AABBRR)			
Asp	3.75	4.39	2.42	6.25
Thre-Asp	9.77	8.83	5.64	10.93
Glut	32.45	28.79	39.81	17.75
Prol	14.49	15.03	16.83	15.29
Gly	8.72	5.77	3.04	6.31
Alan	4.81	4.75	3.09	5.59
Val	5.25	5.30	4.64	6.37

protein and in the protein of common wheat (AABBDD), a high incidence of deviation from the levels of amino acids in the protein of durum (AABB) is evident.

The water-soluble protein fraction of *Triticale* showed significantly higher levels of the amino acids proline and isoleucine. Aspartic acid and alanine levels were lower than in either of the two parent species. The levels of the other amino acids were intermediate between those of the two parental species. Again, a high incidence of deviations of amino acid frequencies was noted in the *Tritipyron* and common wheat proteins when compared to the common durum (AABB) genome.

Examination of the amino acid levels in the unfractionated protein indicates that this approach will not signify differential effects of the alien genomes when considering the relative ratio in which incorporation of the different amino acids occurs. A general "hybrid" or averaging effect (Table II) may be noted with possibly two exceptions—the levels of leucine and isoleucine are higher in *Triticale* than in durum or in rye.

The data presented in Table I show that the combination of two alien genomes results not only in an altered gel electrophoresis pattern as found in earlier studies (*10*) but also in alterations of the amino acid frequencies in the protein fractions. In the case of *Triticale* (AABBRR), most individual amino acid levels are intermediate (averaged or "additive") when compared with the amino acid levels in the parents, rye (RR) and durum (AABB). These observations may be interpreted as due to incomplete dominance of either parental genome, thus giving rise to an averaged incorporation level of the particular amino acid. Where the amino acid frequency is comparable to one or the other parent, this may be interpreted as simple dominance of one of the parental genomes. Where the amino acid frequency is significantly higher than the parent possessing the higher frequency, or significantly lower than the parent with the lower frequency, these present a more complex genetic or amino acid coding problem. It could be suggested that here we have superdominance in which a superimposed coding system has resulted and is expressing itself. This would not be too difficult to visualize for the significantly higher amino acid frequency. In the case of the significantly lower amino acid frequency, the same mechanism could not be operative. Here it is possible that part of the coding system is rendered nonfunctional in some manner (incomplete sequence, interference, etc.).

Examination of the amino acid incorporation data for the other synthetic species, *Tritipyron* (AABBEE), indicates that a similar pattern would evolve if the pattern for the EE parent (*Agropyron elongatum*) were known. It is obvious that the effect of the EE genome is as great in the AABBEE combination as is the RR genome in the AABBRR.

Of considerable interest from an evolutionary standpoint is the amino acid composition of the proteins of common wheat (AABBDD). The deviations from the historical parent (AABB) may be ascribed to the influence of the DD genome. However, in this case, the two species have had hundreds of years in which to diverge, so that "extraction" of the AABB component from common wheat (AABBDD) could conceivably result in a pattern different from the present AABB species (durum wheat). Kerber (*5*) has extracted the AABB component and found morphological differences. Conversely, a synthesis of AABBDD, which has been accomplished, may result in a different amino acid frequency than that found for the genetically "stabilized," natural common wheat (AABBDD).

Comparisons of the amino acid composition of the four protein fractions of each species (Table III) show rather significant differences in amino acid frequencies. One of the most striking differences is apparent when the levels of the basic amino acids (lysine, histidine, and arginine) of the alcohol fraction of rye are compared to the respective levels in water-, salt-, and acid-soluble proteins of the same species. Although the differences in the relative levels of the other amino acids in a similar comparison may not be so striking, they are in a large number of cases significant (beyond experimental error range of the amino acid analyzer) and too numerous to mention specifically. These observations, therefore, further suggest that the fractionation procedure used here and elsewhere has at least some chemical and physical basis. Differences in the amino acid composition of the proteins could conceivably alter the relative charge density, which would give rise to the different protein bands observed in starch-gel electrophoresis studies (*10*), assuming that molecular size does not interfere significantly in specific comparisons. Since gel electrophoresis mobility of a protein is a function of both molecular size (gel network acting as a molecular sieve) and charge density, the contribution to mobility by the two parameters cannot be assessed without molecular weight data—e.g., ultracentrifuge.

Composition of Protein Fractions (Mole Per Cent)

Amino Acid	Acid-Soluble	Salt-Soluble	Alcohol-Soluble	Water-Soluble
Protein Fractions from Triticals (AABBRR)				
Cyst	1.93	3.18	2.05	2.83
Meth	1.40	1.43	1.29	1.47
Isol	4.12	4.12	4.69	4.81
Leuc	7.03	6.43	6.29	6.37
Tyro	3.11	1.87	1.83	1.91
Ph Al	3.91	3.07	4.25	3.59
Lys	2.03	2.03	0.63	2.91
Hist	1.95	2.09	1.58	2.25
Arg	2.33	2.86	1.79	5.27
Protein Fractions from Tritipyron (AABBEE)				
Asp	3.11	4.51	2.29	7.36
Thre-Ser	8.11	9.57	10.97	12.71
Glut	33.27	28.53	23.84	15.65
Prol	14.29	11.79	15.63	13.23
Gly	6.23	5.56	4.41	6.79
Alan	3.68	4.99	3.36	6.90
Val	4.41	5.41	6.52	7.45
Cyst	2.21	4.55	2.68	4.00
Meth	1.39	2.13	1.83	1.62
Isol	3.69	3.47	6.38	4.81
Leuc	6.41	6.28	7.83	6.95
Tyro	2.81	2.53	2.91	2.18
Ph Al	3.99	3.13	5.25	2.87
Lys	1.55	1.91	1.31	3.09
Hist	1.71	1.76	2.10	1.95
Arg	2.69	3.79	2.80	2.37
Protein Fractions from Common Wheat (Kharkov) (AABBDD)				
Asp	2.49	5.39	2.24	10.03
Thre-Ser	8.93	9.82	6.20	9.18
Glut	31.75	23.07	42.78	20.46
Prol	16.91	11.07	16.27	9.00
Gly	6.85	6.99	2.63	7.63
Alan	3.63	6.43	2.61	8.18
Val	4.35	6.10	3.40	5.76
Cyst	1.93	3.71	2.01	4.17
Meth	1.22	1.71	1.15	1.50
Isol	3.69	3.90	4.05	3.32
Leuc	6.34	7.14	6.21	6.33
Tyro	2.63	2.42	1.94	2.28
Ph Al	3.93	3.49	4.71	2.89
Lys	1.58	2.64	0.54	3.15
Hist	1.74	2.23	1.60	1.71
Arg	1.77	3.80	1.62	4.33

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RESIDUE DETERMINATION

A Thin-Layer Chromatographic Procedure for the Determination of Hydrocortisone Acetate and Alcohol Residues in Milk

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A combination thin-layer chromatographic-colorimetric procedure is presented for the determination of hydrocortisone alcohol and acetate residues in milk. After extraction of the steroid from the milk with a methylene chloride–hexane solvent mixture, the residue obtained on evaporation is subjected to thin-layer chromatography to separate the hydrocortisone derivatives. The zones are extracted from the adsorbent and the steroids determined colorimetrically by the Porter-Silber reaction with phenylhydrazine. Control and recovery analyses, carried out by this procedure, gave satisfactory results at all levels investigated with a limit of sensitivity of 10 p.p.b. Recoveries averaged 85.6% for the acetate and 75.1% for the alcohol at levels of 0.010 to 10.00 p.p.m. with control values of zero p.p.b. Related corticosteroids have also been separated by this chromatographic procedure.

Interest in the use of hydrocortisone derivatives as anti-inflammatory agents for the treatment of mastitis in dairy cattle prompted an investigation for the determination of these compounds in milk at the parts-per-billion (p.p.b.) levels. Methods available for the determination of hydrocortisone residues (*1, 4*) were investigated, and they were found inadequate in eliminating interferences of milk background or too time-consuming to be of practical use.

This report deals with the development of a reproducible, specific, and quantitative method for the determination of hydrocortisone derivatives in milk at parts-per-billion levels. The method utilizes a solvent extraction system that does not yield emulsions, with a recovery of 85 to 95% of the solvent; a separation of the hydrocortisone derivatives by thin-layer chromatography; and measurement of the separated components using the color reaction described by Porter and Silber (*3*).

Experimental

Reagents. Purified Methylene Chloride. Slurry 25 grams (approximately 150 ml.) of Nuchar C-190N activated carbon with 1 gallon of methylene chloride. Stir for 15 minutes using a mechanical stirrer. Filter through Whatman #12 fluted filter paper, or its equivalent. All of the methylene chloride used in this procedure must be purified in this manner.

Solvent Mixture. Mix four parts of purified methylene chloride with one part of ACS grade *n*-hexane.

Sulfuric Acid–Ethanol Solvent. Mix two parts of 64% sulfuric acid in water with one part of absolute ethanol.

Phenylhydrazine Hydrochloride Reagent. Dissolve 50 mg. in 50 ml. of sulfuric acid–ethanol solvent. This reagent must be prepared fresh daily.

Silica Gel G and HF_{254}. Distributed by Brinkman Instruments, Inc., Great Neck, L. I., N. Y. Prepare 22 2 × 8 inch plates with a layer of adsorbent 0.25 mm. thick as follows: Mix thoroughly 20 grams each of Silica Gel G and HF_{254}. Add 80 to 90 ml. of distilled water and shake vigorously for 30 seconds. Transfer to the adsorbent applicator and spread rapidly over the plates. Allow to air-dry overnight. When dry, the plates should be scored so that there are five strips on each plate, each strip 1 cm. wide (refer to Figure 1).

Steroid Standards. Obtainable from: U.S.P. Reference Standards, 46 Park Ave., New York 16, N. Y. Weigh accurately 50 mg. of steroid and transfer to a 50-ml. volumetric flask. Dissolve in absolute ethanol, dilute to volume, and mix well. Label Solution A. Pipet 10 ml. of Solution A into a 100-ml. volumetric flask and dilute to volume with ethanol. Mix well and label Solution B. Pipet 10 ml. of solution B into a 100-ml. volumetric flask and dilute to volume with absolute ethanol. Mix well and label Solution C. Pipet 10 ml. of Solution C into a 100-ml. volumetric flask and dilute to volume with absolute ethanol. Mix well and label Solution D. This is the working standard containing 1.0 μg. of steroid per ml. of solution.

Apparatus. Funnels, separatory, 1000-ml., 125-ml., and 60-ml. capacity with polytetrafluoroethylene stopcocks.

Chromatographic equipment (Desaga), thin-layer, with 2 × 8 inch plates. Flasks, acetylation, 10-ml. capacity, Kimble Cat. No. K29425. Absorption cells, Arthur H. Thomas, Cat. No. 9102-M80, 0.7-ml. capacity, 1-cm. path length.

Vibrating shaker, Research Specialties Co., Richmond, Calif.

Analytical Procedure

Calibration Curve. Transfer aliquots of standard Solution D containing 2, 4, 6, 8, and 10 μg. of steroid to test tubes and evaporate to dryness under a stream of air in a water bath at 55° C. Using an