

A STUDY OF THE ASSAY METHOD FOR THE GUINEA PIG ANTISTIFFNESS FACTOR*

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In 1941 Wulzen and Bahrs (1) reported a characteristic muscular stiffness at the wrist among guinea pigs on a skim milk diet, which progressed in intensity over a period of months. Autopsies of deficient animals revealed extensive calcification throughout the muscles and internal organs. Raw cream fed by mouth was found to alleviate the wrist stiffness within a few days, although disappearance of calcium phosphate deposits required many months.

A procedure for the isolation of a factor present in raw cream which cured an induced stiffness in guinea pigs was described in 1943 (2). 3 mg. of the factor were isolated from 15 gallons of raw cream. The compound was reported to be an oily ketone (steroid) which was very susceptible to atmospheric oxidation and was active in dosages of 0.1 γ .

The factor present in cream was later traced to the molasses which had been added to the diet of the cow, increasing thereby the original amount in cream. This led to the use of molasses and finally to unheated cane juice as a source of the factor. The procedure for the isolation from molasses or cane juice has been reported (3). 100 mg. of a factor (pure white leaflets, m.p. 81.5–82°) were isolated from 55 gallons of cane juice, which was very susceptible to atmospheric oxidation and was active in dosages of 0.002 γ (3).

Three reviews (4) have pointed out the need for the confirmation of this work by other laboratories, as well as information regarding the reliability and limitations of the assay. Experiments of this nature have been published recently by Oleson and coworkers of the Lederle Laboratories (5). These have confirmed the existence of a stiffness syndrome, as well as the curative power of a crystalline material derived from cane juice, when fed in a 5 γ dosage. Furthermore, several compounds related to the common sterols were reported which exert a similar curative effect.

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Since the work on the isolation and identification of the natural factor at Oregon State College was interrupted,¹ experiments were undertaken by the authors to confirm the earlier work and carry the problem to completion. Samples of molecularly distilled cane wax (equivalent when first made to that reported as containing 10,000,000 units of activity per gm. of material (3), curative in 0.1 γ dosage) were obtained for this purpose from Eli Lilly and Company.² However, when this material was separated into various fractions by means of a flowing chromatograph, it was not possible to follow further concentration changes. The results from the assay were frequently contradictory and the precision was so low that further concentrations of the active principle could not be followed with any degree of certainty.

Although the assay has evidently been adequate to establish the presence or absence of the syndrome, additional evidence is required to fix its limitations and to ascertain whether it can serve as an analytical tool for isolation of the natural factor. Since this is a crucial matter, a careful check of the assay procedure was undertaken.

This problem was approached by dilution experiments as well as other treatments. Samples of the wax were prepared in various dilutions. These were assayed and the results clearly indicate no differentiation among animals fed wax over a several fold concentration range. They confirm the Lederle experiments (5) which show that there is no significant difference in animals fed the 1 and 5 γ dosages.

The wax was also subjected to a number of treatments (adsorption, molecular distillation, treatment with pure oxygen at 100° for 24 hours, removal of the ketone fraction, fractional crystallization, etc.) which should yield fractions with varying degrees of potency. These materials, together with a number of other samples (*i.e.* ertron, the Lilly factor, an active sterol (Lederle), and Cuban cane wax)² which for various reasons were thought to possess marked activity, were submitted to assay. The identities of all samples were unknown to the analyst at the time the assays were performed. All the data obtained over a period of 1 year, in which duplicate assays (in groups of three to eight animals) were made, are tabulated in Table I.

If the method has quantitative value, there should be significant differences among the assays of the animals receiving the different treatments.

¹ Due to the resignation of Dr. W. J. van Wagendonk to accept a position at the University of Indiana.

² The authors are indebted to Dr. A. L. Caldwell of Eli Lilly and Company for the samples of cane wax and the highly active component of cane wax, to Dr. A. J. Kunschner of the Nutrition Research Laboratories for samples of ertron, and to Mr. George P. Van Dooren for samples of Cuban sugar-cane wax.

TABLE I
Assays on Materials Used in Treatment of Cane Wax

Group No.	Treatment No.	Material tested	Dosage	Change per animal unit*
			γ	
70		Negative control		-0.1
71		“ “		-0.3
75		“ “		-0.3
77		“ “		0.8
82		“ “		0.0
89		“ “		0.0
103		“ “		0.1
107		“ “		-0.1
112†		“ “		0.7
157		“ “		0.0
163†		“ “		1.3
172		“ “		0.7
119	1	Lederle sample	100	-0.1
173	1	“ “	100	0.8
183	1	“ “	100	0.0
174	2	“ “	5	0.2
176	2	“ “	5	0.1
			<i>mg.</i>	
143	3	FA-5	6	0.0
165	3	“ “	6	0.8
161	4	FB-1	6	0.4
155	4	“ “	6	0.6
102	5	Wax 2	6	1.2
116	5	“ 2	6	1.9
184	5	“ 2	6	1.0
74	6	Ketones, Wax 2	1	1.0
90	6	“ “ 2	1	0.0
134	7	“ “ 2	6	1.8
139	7	“ “ 2	6	1.4
162	7	“ “ 2	6	0.4
168	7	“ “ 2	6	0.0
135	8	Non-ketones, Wax 2	6	0.0
140	8	“ “ 2	6	1.0
136	9	Adsorbed, Wax 2	6	0.0
142	9	“ “ 2	6	0.5
133	10	Unadsorbed, Wax 2	6	0.8
141	10	“ “ 2	6	0.2
169	11	Fraction FB-6	6	0.0
159	11	“ “	6	0.7
76	12	Cuban wax	1	1.3
93	12	“ “	1	0.1
78	13	Ertron	1	1.6
94	13	“	1	1.7
166	14	O ₂ , 24 hrs., 100°†	6	1.0
170	14	“ 24 “ 100°	6	0.2
171	14	“ 24 “ 100°	6	1.6

TABLE I—*Concluded*

Group No.	Treatment No.	Material tested	Dosage	Change per animal unit*
			<i>mg.</i>	
175	14	O ₂ , 24 hrs., 100°	6	0.0
B	15	Wax 5	10	0.0
E	15	" 5	10	0.0
G	15	" 5	10	0.0
128	16	" 5	6	2.1
137	16	" 5	6	1.2
147	16	" 5	6	-0.1
A	17	" 5	1	0.0
C	17	" 5	1	0.2
D	17	" 5	1	0.0
F	17	" 5	1	0.5
125	18	" 3A	6	1.2
138	18	" 3A	6	0.3
148	18	" 3A	6	1.9
I	19	Lilly factor	0.1	0.3
J	19	" "	0.1	-0.3
122	20	Wax 2 (new)	6	-0.2
185	20	" 2 "	6	1.1
157	21	Fraction FB-4	6	1.5
177	21	" "	6	0.2

* The total change with the 1 to 4 system, described previously (3), is given as the sum of the changes in the readings of left and right paws for all animals over a period of 1 week. A change in reading from 3 to 4 in one paw, for example, corresponds to 1 unit.

† Negative controls, Groups 112 and 163, contained 0.2 to 2 mg. of fat acid.

‡ Wax 2.

In order to determine this a statistical analysis³ was made of all the tests in Table I, the negative controls and the animals for which the reading in either paw was missing being excluded. In all, the statistical study deals with twenty-one treatments and 53 groups of animals, consisting of 162 individuals. The results of the analysis are shown in Table II.

Table II gives three sources of variation; namely, (a) the variation among the different treatments, (b) the variation among the groups which received the same treatment, and (c) the variation among the animals which happened to be in the same arbitrary group. The three variances listed in Table II are the respective quantitative measures of these variations. The ratio of the group variance to the animals' variance is given in Table II as 3.40. As the 1 per cent level of Snedecor's *F* distribution, with 32 and 109

³ Statistical treatment of the experimental data was carried out by Dr. Jerome C. R. Li of the Oregon State College Mathematics Department. The authors wish to express their gratitude for his helpful suggestions.

degrees of freedom, is less than 1.89, this variance ratio, 3.40, indicates that the variation among the groups is significantly greater than that among the animals within each group, despite the fact that the animals were arbitrarily divided into different groups.

There is no obvious factor which should cause the various groups to react differently to the same treatment. Some factor not fully controlled such as the incidence of infectious disease in the animal colony or deterioration of the physiological activity of the specimens with time may have contributed to statistical uncertainty. Whatever the reason may be, it illustrates the inadequacy of the method for quantitative assay purposes in its present form.

The variance 1.4302 due to the treatments is approximately equal to the variance 1.3796 due to the groups which receive the same treatment. The variance ratio is 1.04. This clearly indicates that the different treatments

TABLE II
Analysis of Variance

Sources of variation	Degrees of freedom	Sum of squares	Variance	Variance ratio
(a) Treatments	20	28.6030	1.4302	3.40
(b) Groups within treatment	32	44.1461	1.3796	
(c) Animals within group	109	44.2509	0.4060	
Total.....	161	117.0000		

do not show different effects in this experiment and that it is not possible to differentiate among animals fed samples of materials from cane wax and other sources.

DISCUSSION

Since the data presented here, together with those from the Lederle report, indicate that it is not possible to make comparisons of relative activity with stiffness measurements within a several fold concentration range, it seems doubtful whether any concentrations other than an almost perfect separation, such as removal of wax from cane juice by ether extraction, could be followed. The distribution of active materials resulting from the treatment of cane wax either by chromatography, molecular distillation, or fractional crystallization would hardly be likely to occur in such favorable ratios as to permit differentiation between various fractions by this assay procedure. Considering (a) the fact that Oleson and co-workers have shown a number of common sterols to possess antistiffness activity, (b) the low sensitivity of the assay, and (c) the distribution usually

effected in isolation work, it does not seem strange that all the fractions isolated in this laboratory appeared relatively equal in activity and that differentiation between them is an impossibility.

SUMMARY

The assay procedure (2, 3) for the guinea pig antistiffness factor has been studied with respect to its reproducibility and sensitivity of measurement over a several fold concentration range of active sugar-cane wax. Statistical analyses of the results indicate that there are no significant differences among samples of wax receiving different chemical treatments. It is concluded *that the assay procedure is not suitable for quantitative analytical purposes.*

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