Thermal stability of sodium hyaluronate in aqueous solution

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Since its identification 60 years ago as a ubiquitous component of the body of mammals, hyaluronic acid has been widely studied, primarily in the fields of medicine and biology. On the other hand, our research has dealt with hyaluronic acid as a chemical intermediate in the synthesis of novel lubricious coatings, and in this connection data were needed on stability of aqueous solutions of the polymer over a range of temperatures from 25–100°C. The investigation reported here provides that information, obtained by exposing samples in sealed ampules in baths at controlled temperatures and determining the resulting change in viscosity of the solutions. Data of this kind have not previously been reported on sodium hyaluronate freed from the proteins and other organics normally associated with the polymer in its natural environment. © 1994 John Wiley & Sons, Inc.

INTRODUCTION

Hyaluronic acid and its salts are ubiquitous in mammalian bodies, serving in aqueous solution as lubricants, as viscoelastic humectants, in wound healing, and in various physiologic roles.[1–5] It has never been synthesized *in vitro*, and was first isolated in 1934 by Meyer and Palmer[6] from bovine vitreous humor. It has since been isolated also from umbilical cord, rooster comb, and bacterial culture broths. In all of its natural sources, the acid and salts are closely associated with other carbohydrates, proteins, lipids, and unidentified impurities, and over the years of its recognition as a polymeric "entity," the problems of isolating and purifying the substance have been studied and processes refined.[7–9]

Partly because of its polymeric nature, but mainly because of its biologic importance, sodium hyaluronate (NaHy) has been given greater attention in the fields of medicine and biology than by chemists. In our research of the past 10 years, however, its role has been that of a most unusual and valuable chemical intermediate in the synthesis of novel, lubricious coatings.[10–18]

In the course of that research, the need arose early for reliable information on the thermal stability of aqueous solutions of NaHy. Published information on the subject proved not to be amenable to comparison on a common basis, especially not for establishing kinetics of the degradation over a range of temperatures of interest. Furthermore, uncertainty about the purity of the polysaccharide used in studies before the past decade made the data unsuitable except in the most general sense.

In this study, the viscosity (a function of molecular weight) of dilute aqueous solutions of an ultrapure grade of NaHy was monitored after controlled times of exposure to various temperatures ranging from 25–90°C in sealed containers.

TABLE I
Analytical Values for Sodium Hyaluronate, Pharmaceutical Grade

Appearance	White, odorless powder
Sodium chloride content	0.14%
Sodium hyaluronate content	93%
Sodium content	5.2%
Water content (Karl Fischer)	10.7%
Intrinsic viscosity	1970 ml/mg
Molecular weight	1.3×10^6 d
pH (1% aqueous solution)	6.9
Proteins	0.05%
Sulfated ash	16.1%
Viable count	0/g
Salmonella	Negative
Escherichia coli	Negative

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TABLE II
Experimental Data

Series 1

50°C					60°C				
Time	CFT	s^2	Ratio	$s^2 \times 10^5$	Time	CFT	s^2	Ratio	$s^2 \times 10^5$
0.0	317.56	0.1876	1.000	0.3721	0.0	317.56	0.1876	1.0000	0.3721
0.5	303.18	1.5567	0.9550	1.7130	1.0	370.60	42.8895	1.1670	42.7800
1.0	304.59	0.2680	0.9500	4.3690	2.0	355.73	0.7358	1.1202	0.9631
2.0	337.81	5.0330	1.0640	5.2010	3.0	332.33	30.2323	1.0465	30.1800
4.0	315.97	2.5658	0.9950	2.7280	4.0	297.20	75.0236	0.9359	74.5600
6.0	316.12	4.1398	0.9950	4.2890	5.0	325.94	20.0130	1.0263	20.0400
7.7	307.11	1.8452	0.9670	2.0040	24.0	301.90	3.1650	0.9507	3.3070
24.0	302.53	0.6268	0.9530	0.7904	50.5	266.83	7.3560	0.8403	7.4240
48.0	312.46	0.4024	0.9840	0.3996					

Series 2

50°C					70°C				
Time	CFT	s^2	Ratio	$s^2 \times 10^5$	Time	CFT	s^2	Ratio	$s^2 \times 10^5$
0.0	430.71	0.4212	1.0000	0.4541	0.0	430.71	0.4212	1.000	0.4541
1.0	516.86	6.9854	1.2000	4.0920	1.0	509.98	9.4345	1.1840	5.4040
2.0	545.46	0.4645	1.2664	0.6145	2.0	485.22	0.8233	1.1266	0.7320
4.0	535.07	6.7544	1.2423	3.9910	4.0	504.45	1.6825	1.1712	1.2180
6.0	421.50	1.7100	0.9786	1.1390	6.0	381.37	0.1873	0.8854	0.2790
24.0	498.70	7.6750	1.1579	4.4420	24.0	502.65	0.3925	1.1670	0.5208
48.0	447.67	9.1033	1.0394	5.1520	48.0	284.85	0.0616	0.6613	0.1325
96.0	485.71	0.5130	1.1277	0.5653					

Series 6

50°C					60°C				
Time	CFT	s^2	Ratio	$s^2 \times 10^5$	Time	CFT	s^2	Ratio	$s^2 \times 10^5$
0.0	2308	223.0	1.0000	83.5	0.0	1055	79.8	1.0000	14.3
0.5	2502	474.9	1.0841	58.0	1.0	1153	495.7	1.0929	53.1
1.0	2332	4090.0	1.0104	119.4	2.0	1080	2.6	1.0237	7.7
2.0	2318	877.0	1.0043	58.6	3.0	1152	495.7	1.0919	53.1
4.0	2485	8164.0	1.0767	201.6	5.0	1363	791.3	1.2919	83.1
6.0	2538	5022.0	1.0997	144.7	72.0	746	9.4	0.7545	4.9
24.0	2168	364.0	0.9393	43.7	96.0	636	32.8	0.6028	5.6
48.0	1883	82.3	0.8159	29.3					
54.3	2033	12441.0	0.8808	265.9					

90°C				
Time	CFT	s^2	Ratio	$s^2 \times 10^5$
0.0	1055	79.8	1.0000	14.3
0.5	1015	16349.0	0.9621	1476.0
1.3	775	66.6	0.7346	9.9
2.0	640	58.8	0.6066	7.9
4.0	203	3.2	0.1924	0.6
6.0	374	13.6	0.3545	2.1
24.0	239	20.8	0.2265	2.2

MATERIALS AND METHODS

The NaHy used was of an ultrapure pharmaceutical grade, supplied by the Pharma Division of Diosynth, with the properties shown in Table I. Two liters of an 0.03% (wt/wt) solution in distilled water was prepared, filtered through Whatman no. 50 paper, and stored in a closed container in a refrigerator at temperatures ranging from just above freezing to 5°C. Under these experimental conditions, growth of microorganisms was inhibited.

Fifteen-milliliter aliquots of this solution were pipetted into glass ampules and the necks sealed with oxygen torch. The desired number of sealed ampules were immersed in a stirred oil bath set at the temperature of interest and controlled to within ±0.5°C. At the end of the desired exposure intervals, one or more ampules were removed, immediately immersed in ice-water for 5 min, and then transferred to the refrigerator. The ampules remained in the refrigerator for approximately 15 min and were then removed one at a time and broken at the score, and the solu-

TABLE II
Continued

Series 1					Series 2				
		70°C					25°C		
Time	CFT	s^2	Ratio	$s^2 \times 10^5$	Time	CFT	s^2	Ratio	$s^2 \times 10^5$
0.0	317.56	0.1876	1.0000	0.37	0.0	534.07	5.5297	1.0000	3.8770
0.5	340.54	4.2314	1.0724	4.41	2.0	529.08	0.0809	0.9907	1.9310
1.0	377.90	151.006	1.1900	150.00	4.0	521.82	24.8954	0.9771	10.0580
2.0	340.36	108.650	1.0718	108.00	23.3	518.38	10.0093	0.9706	5.3360
					28.3	517.68	14.2699	0.9693	6.8240
					46.3	514.22	46.4422	0.9628	18.0800
					75.0	513.61	0.6242	0.9617	2.0120

Series 2					Series 3				
		90°C					70°C		
Time	CFT	s^2	Ratio	$s^2 \times 10^5$	Time	CFT	s^2	Ratio	$s^2 \times 10^5$
0.0	430.71	0.4212	1.0000	0.4541	0.0	484.24	43.579	1.0000	37.170
1.0	428.44	0.4900	1.0179	0.4994	1.0	467.07	0.826	0.9645	17.640
2.0	258.04	0.0032	0.5991	0.0832	2.0	475.50	0.426	0.9820	18.100
4.0	318.41	0.1540	0.7393	0.2071	4.0	481.18	0.410	0.9937	18.530
6.0	279.93	0.0456	0.6499	0.1205	7.0	418.01	7.717	0.8632	17.140
24.0	122.08	0.1477	0.2834	0.0979	12.0	385.15	0.260	0.7954	11.870
48.0	95.94	0.0481	0.2227	0.0372	24.0	375.24	0.071	0.7749	11.190
					28.0	351.41	0.320	0.7257	9.924

Series 6									
		70°C					80°C		
Time	CFT	s^2	Ratio	$s^2 \times 10^5$	Time	CFT	s^2	Ratio	$s^2 \times 10^5$
0.0	1055	79.8	1.0000	14.3	0.0	1055	79.8	1.0000	14.3
0.5	1353	12649.0	1.2825	1148.0	1.0	1070	2054.7	1.0142	192.0
1.0	1233	250.7	1.1687	32.3	2.0	922	232.9	0.8739	26.4
2.0	1263	1597.0	1.1972	153.8	3.0	903	106.6	0.8559	14.8
4.0	1032	38.8	0.9782	10.3	4.0	766	10.1	0.7261	4.7
5.5	1085	1637.0	1.0284	154.6	5.0	855	602.1	0.8104	58.8
24.0	752	166.7	0.7128	18.6	24.0	541	255.0	0.5128	24.8
					48.0	306	465.1	0.2900	42.4

tion was filtered through a 30-fine fritted glass disk. A 10-ml portion of the filtrate was pipetted into the viscometer mounted in a constant-temperature bath at 25°C. After a period of 30 min to allow equilibration to the test temperature, capillary flow times (cft) were determined in calibrated Cannon-Fenske Routine Viscometers. Three such observations were made for each sample, and the values were averaged (Table II). The flow times (seconds) could be converted to specific viscosities by using the cft for water; however, because our interest is in the change of viscosity as a function of exposure time, we analyzed the following quantities instead:

$$r = \frac{cft_t}{cft_0}$$

$$s_r^2 = \frac{1}{cft_0^2} s_{cft_t}^2 + \frac{cft_t^2}{cft_0^4} s_{cft_0}^2.$$

In these equations cft_t is the cft after exposure time of t h, cft_0 is the cft at zero exposure time, and s^2 is the sample variance of the subscripted observation.

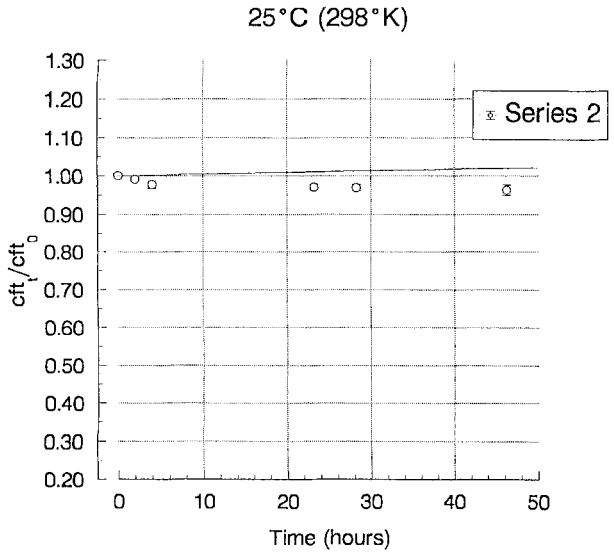

Figure 1. Change of relative viscosity at 25°C.

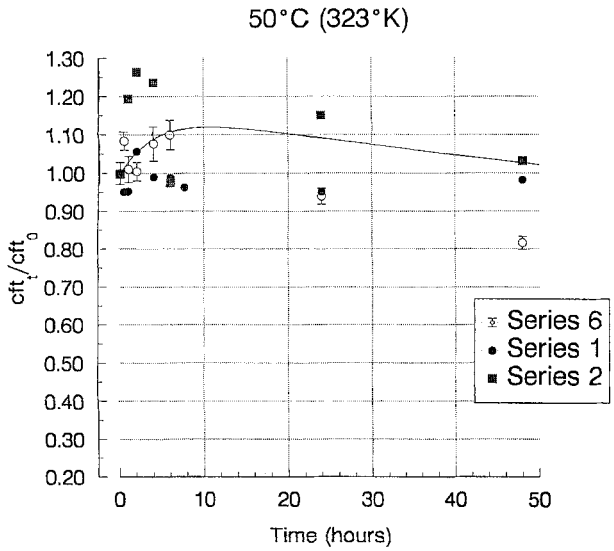

Figure 2. Change of relative viscosity at 50°C.

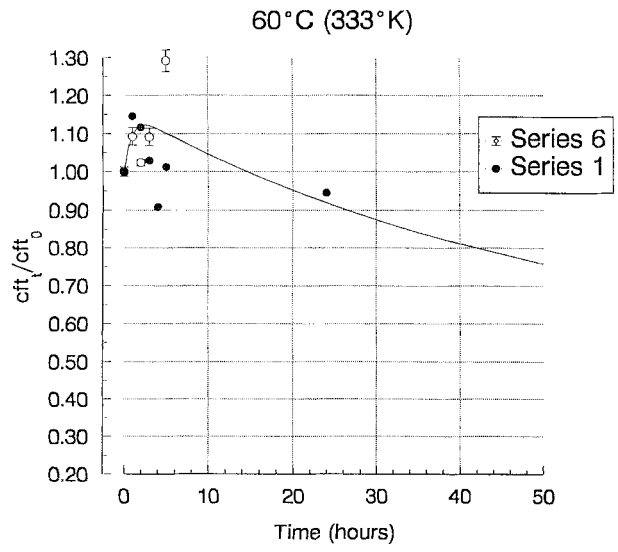

Figure 3. Change of relative viscosity at 60°C.

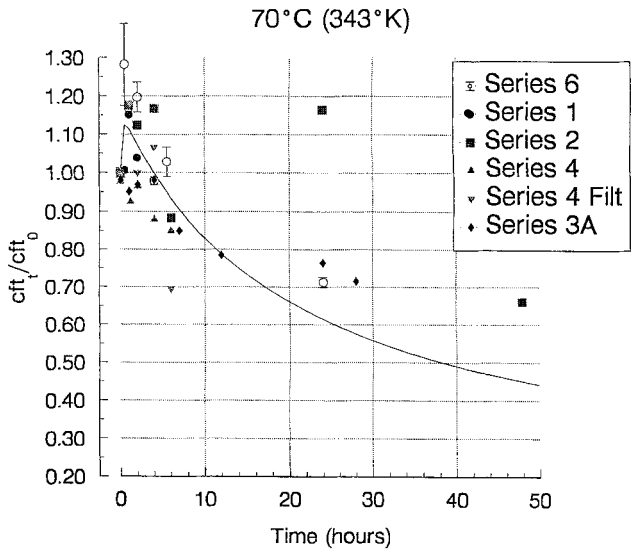

Figure 4. Change of relative viscosity at 70°C.

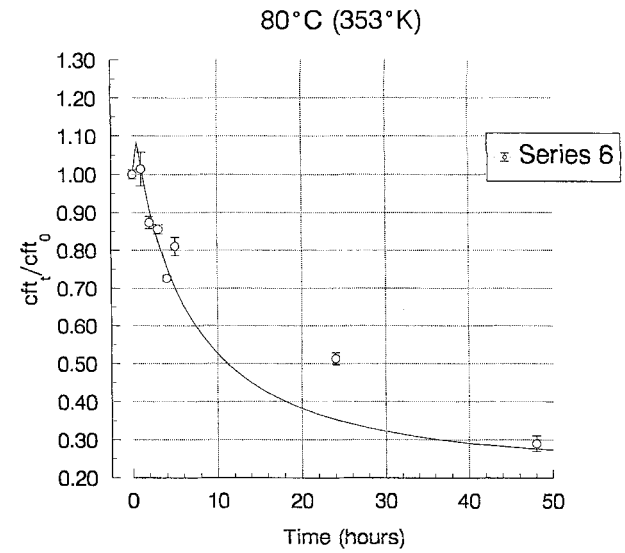

Figure 5. Change of relative viscosity at 80°C.

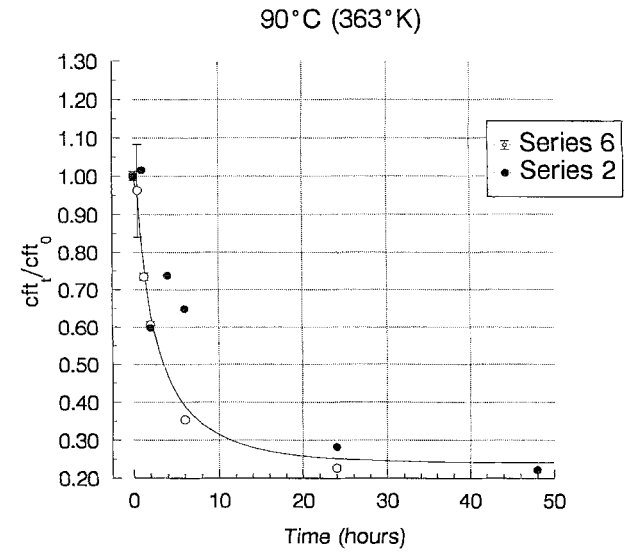

Figure 6. Change of relative viscosity at 90°C.

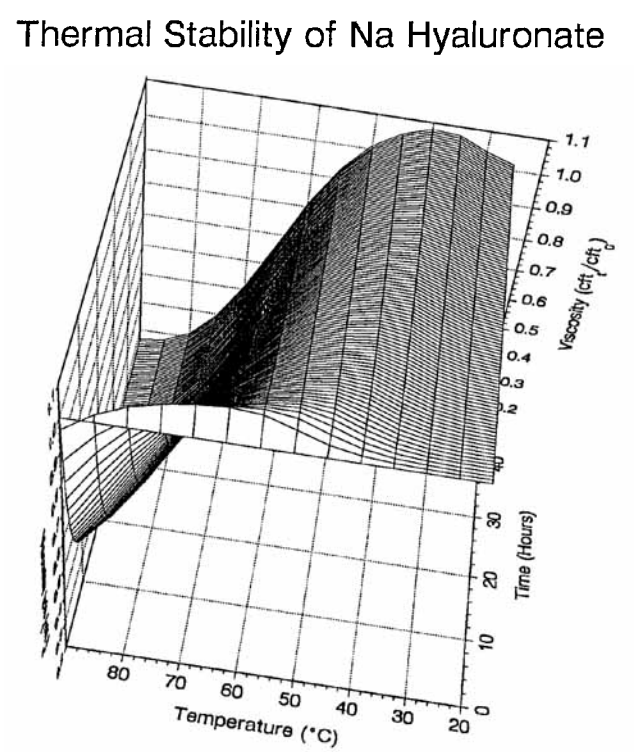

Figure 7. Time of exposure relative to temperature and viscosity.

The data were fitted to a model by means of an iterative numerical technique with use of the software "TK Solver Plus[R]" supplied by Universal Technical Systems, Inc. The actual and fitted data at the temperatures studied are shown in Figures 1 through 6. Error bars are shown for one of the data series (series 6) but not for others because the limitations of monochromatic printing would not allow one series to be distinguished from another. With use of the fitted model, Figure 7 is constructed to relate time of exposure to temperature and to viscosity. Figure 8 is a topographic map of Figure 7.

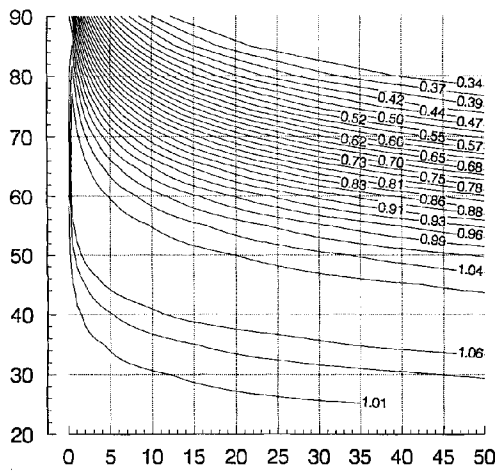

Figure 8. Topographic map of stability data.

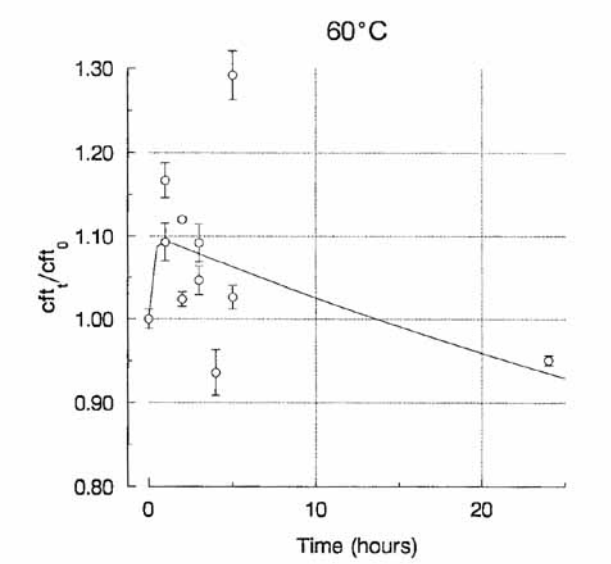

Figure 9. Illustration of initial increase in viscosity.

DATA ANALYSIS

It is apparent in examining the data that the NaHy solution seemed to undergo a significant increase in viscosity for a period of time before the decline in viscosity associated with polymer chain degradation at all temperatures higher than 25°C. This is illustrated in Figure 9 for data at 60°C, where the length of the error bars corresponded to 1 SD on either side of the average for the point. If only thermally induced chain scission were taking place, none of the plotted numbers would be greater than 1.

This phenomenon, if real, may be related to the well-known viscoelastic behavior of solutions of NaHy of molecular weight higher than about 1,000,000,[19,20] which has been attributed to extensive

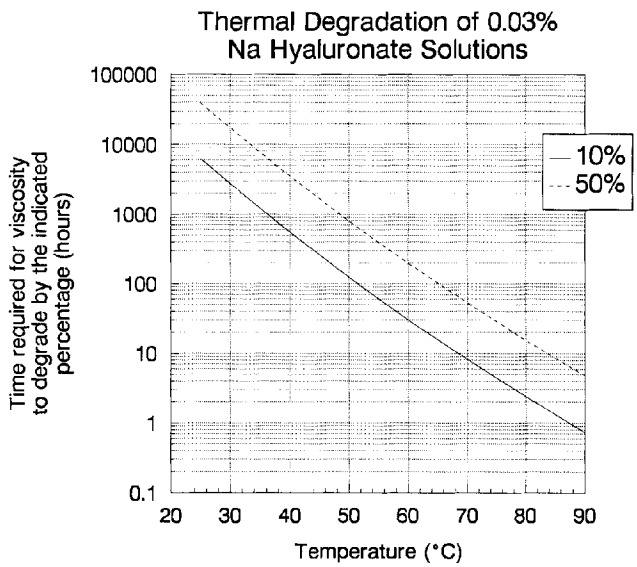

Figure 10. Time required for reduction in viscosity by 10% and by 50% from initial values.

chain entanglement and chain–chain interaction of the highly hydrated polymer.[21] On the other hand, we tend to favor the idea that temperatures higher than that at which the original dry polymer was put into solution induce further "dissolving"—that is, further separation of chain segments hydrogen-bonded to each other as in the original solid. The effect on viscosity is tantamount to increasing the number and/or length of polymer molecules. The additive effect would then be overtaken and decline as simultaneous chain scission due to thermal degradation continues at rates characteristic of the temperature.

At 25°C, there was virtually no change in viscosity over the period studied. No net decline was seen at 50°C, but at 60°C and above, there was a rapid increase in viscosity followed by exponential rates of decline. Figure 10 shows the times required for 10 and 50% degradations in viscosity calculated from the fitted data.

CONCLUSIONS

The rate of viscosity breakdown of an aqueous solution of sodium hyaluronate increases exponentially with temperature. At 25°C, a drop in viscosity of 10% requires many thousands of hours; at 90°C, the same drop occurs in <1 h.

The practical conclusion is that NaHy solutions should be stored in the short term at temperatures no higher than 25°C. Storage at 0–5°C would further improve stable life by approximately 2 orders of magnitude, other factors being equal. A sterile solution of NaHy protected from inoculation by microorganisms and stored in the refrigerator is estimated on the basis of these results to be stable for many years.

Mrs. Renee (Luckenbill) Keller contributed to the experimental work described here. Dr. John D. Guerin (Turning Points Management Consulting, Inc.) supplied much of the data analysis.

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