Stability of Insulin Preparations

M. Pingel, M.Sc., and Aa. Vølund, M.Sc., Lic. Techn., Copenhagen

SUMMARY

The stability of seven types of pharmaceutical insulin preparations was determined by a bioassay (mouse convulsion method) and a radioimmunoassay after storage for different periods at 4° C., 15° C., 25° C., 37° C., or 45° C. in the dark. The seven insulin preparations were: Ordinary Insulin, Protamine Zinc Insulin (PZI), the Lente insulins (Ultralente, Semilente and Lente), Rapitard and Actrapid.

Full biological potency was registered in all the insulin preparations under study after five years of storage at 4° C. The biological potency (P(t,T)) as function of time (t) and absolute temperature (T) can be expressed by the formula:

$$P(t,T) = P_0 e^{-te^{(\alpha-\beta/T)}}.$$

Using the values observed, the constants P_0 (initial potency), α, and β were calculated for each type of insulin preparation. According to the formulas the loss in biological potency for all the insulin preparations will be less than 10 per cent after storage for sixty-nine years at 5° C., ten years at 15° C., twenty months at 25° C., three months at 35° C. or ten days at 45° C. The activation energy (E_a), the half-life ($t_{0.5}$) and the temperature coefficient (Q_{10}) were calculated for each of the seven insulin preparations using α and β. The rate of disappearance of biological insulin activity was found to increase four- to fifteenfold with a temperature increase of 10° C.

The decline in immunological activity was less than that in biological activity, especially in cases where the loss in biological potency was substantial. It is concluded that the immunoassay is no reliable substitute for the bioassay of unknown insulin preparations. DIABETES *21*:805-13, July, 1972.

The stability of insulin preparations has been examined by several investigators.[1-7] Most studies[1-4,6] have dealt only with acid insulin solutions. Krogh and Hemmingsen, 1928,[1] were the first to investigate the relations between temperature, time and biological potency of amorphous insulin in aqueous solutions (pH 1-4). They found that the decrease in biological insulin activity at constant temperature follows fairly well the kinetics of a monomolecular reaction. Sahyun et al., 1937, 1939,[2,3] showed that the addition of Zn^{++} to the aqueous medium significantly improved the stability. This could not be confirmed by Lens, 1947,[4] who reported that the stability of crystalline insulin in acid solution was unpredictable. Lens concluded that the inactivation was not due to hydrolysis of the insulin but could be attributed to denaturation, usually followed by an irreversible oxidative process. Schlichtkrull, 1958,[5] investigated the stability of the Lente insulins (Insulin Zinc Suspensions, pH 7.4) and found a first-order reaction for the potency loss at 37° C. and 50° C. Stephenson and Romans, 1960,[6] studied the stability of acid insulin solutions. They state[6] that, during the initial period, the loss in potency may be a reaction of zero order.

Storvick and Henry, 1968,[7] used a radioimmunoassay to determine the stability of pharmaceutical insulin preparations. They found[7] all neutral insulin preparations to be more stable than acid insulin preparations. Contrary to the findings of Storvick and Henry,[7] Peck and Schechter, 1944,[8] state that the stability of insulin solutions at pH 7.2 is only about one tenth of that at pH 3, while Rückert and Schöne, 1962,[9] state that insulin solutions containing protamine sulphate have maximum stability at pH 4.0 to 4.5. The reason for these differences probably lies in the fact that some of the older studies used insulin preparations made of impure insulin, possibly containing enzymes, whereas most of the insulin preparations in use today (and for the past twenty years or more) are made of recrystallized insulin.

The present investigation deals with the stability of seven types of pharmaceutical insulin preparations, as determined by a bioassay (mouse convulsion method)

From Novo Terapeutisk Laboratorium and Novo Research Institute, 215 Nordre Fasanvej, DK 2200, Copenhagen N, Denmark.

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/21/7/805/346849/21-7-805.pdf by guest on 30 March 2023

and a radioimmunoassay. On the basis of the results of the bioassay, a formula is derived for each of the insulin preparations, that enables the calculation of biological potency as function of storage time and temperature.

MATERIALS AND METHODS

The study was done on seven different types of the pharmaceutical insulin preparations manufactured by Novo Industri A/S, Copenhagen. The seven insulin preparations were the following:

Ordinary Insulin (Acid solution of pork and beef insulin).
Protamine Zinc Insulin (PZI) (Neutral suspension of amorphous protamine-zinc-insulin complex, pork and beef insulin).
Ultralente (Neutral suspension of beef insulin crystals).
Semilente (Neutral suspension of pork insulin in the amorphous state).
Lente (A mixture of seven parts Ultralente and three parts Semilente).
Rapitard (Neutral suspension containing three parts beef insulin crystals and one part pork and beef insulin in solution).
Actrapid (Neutral solution of pork insulin).

Ordinary Insulin and PZI were made of insulin crystallized at least twice, which had a biological activity of 25.0 U./mg. dry weight ± 2 per cent corresponding to 165 U./mg. N. The dry weight is determined after drying the insulin at 105° C. for eighteen hours. Ultralente, Semilente, Lente, Rapitard, and Actrapid were made of insulin crystallized at least three times, which had a biological activity of 26.1 U./mg. dry weight ± 2 per cent, corresponding to 171 U./mg. N. These figures of biological activity of the recrystallized insulin are the result of biological assays against the Fourth International Standard (mouse convulsion method) performed over five years. The stated potency of the insulin preparations under study was 40 U./ml. or 80 U./ml. calculated on the basis of the biological potency of the insulin used.

Biological insulin determinations were carried out on diluted solutions by the modified mouse convulsion method according to Young and Lewis, 1947.[10] For the preparation of the reference standards, use was made of the Fourth International Standard for insulin. The results were calculated by standard statistical methods for quantal response assays.[11]

Radioimmunological insulin determinations were carried out on diluted solutions by the ethanol precipitation method according to Heding, 1967.[12] For the preparation of the reference standards, use was made of the Fourth International Standard for insulin.

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/21/7/805/346849/21-7-805.pdf by guest on 30 March 2023

TABLE 1

Biological potency in per cent of the stated potency after storage for different periods at 4° C., 15° C. or 25° C. The figures outside the brackets show the experimentally found potencies with 95 per cent confidence limits, whereas the figures inside the brackets are derived from the formulas.

		0 yr.	1 yr.	2 yrs.	3 yrs.	4 yrs.	5 yrs.	8 yrs.	10 yrs.
4° C.	Ordinary insulin	99±2 (97)		94±5 (97)	97±5 (96)	98±16(96)	99±5 (96)		
	PZI	100±3(100)		97±7(100)	105±5(100)		94±5(100)		
	Ultralente	99±3 (96)		97±7 (96)	97±7 (96)		94±5 (96)		
	Semilente	101±2(100)		102±8(100)	102±6(100)		102±9(100)		
	Lente	99±1 (99)		105±9 (99)	106±6 (99)		103±9 (99)		
	Rapitard	100±2(100)		97±8(100)	102±7(100)		101±7 (99)		
	Actrapid	100±3(100)		96±8(100)	101±8(100)		98±6(100)		
15° C.	Ordinary insulin			91±4 (95)	90±4 (94)		90±5 (92)		
	PZI			100±5 (99)	95±5 (99)		97±6 (98)		
	Ultralente			98±8 (94)	88±5 (93)		85±6 (92)		
	Semilente			96±6 (99)	94±7 (99)		89±8 (98)		
	Lente			100±8 (98)	97±8 (97)		89±7 (96)		
	Rapitard		98±19(99)	92±7 (99)	98±6 (98)		98±6 (97)		
	Actrapid			104±8(100)	100±7 (99)		95±8 (98)		
25° C.	Ordinary insulin		95± 8(91)	84±3 (86)	84±3 (81)	69±10(76)	70±3 (71)	58± 8(60)	55±4 (53)
	PZI		105±11(96)	101±6 (92)	91±5 (88)		78±4 (82)	74±12(73)	70±6 (67)
	Ultralente			88±4 (85)	79±5 (80)	77± 8(75)	72±4 (70)	60± 6(58)	49±4 (51)
	Semilente			91±6 (94)	96±6 (91)	86±12(88)	84±3 (85)	76± 7(77)	76±6 (72)
	Lente		93±12(94)	86±7 (89)	87±7 (85)		76±5 (77)	64± 9(66)	62±5 (60)
	Rapitard		96±10(96)	96±7 (93)	87±7 (90)		88±7 (83)	73± 8(75)	
	Actrapid		103± 9(98)	99±7 (96)	94±6 (94)		92±7 (91)	84± 8(86)	

and a radioimmunoassay. On the basis of the results of the bioassay, a formula is derived for each of the insulin preparations, that enables the calculation of biological potency as function of storage time and temperature.

MATERIALS AND METHODS

The study was done on seven different types of the pharmaceutical insulin preparations manufactured by Novo Industri A/S, Copenhagen. The seven insulin preparations were the following:

Ordinary Insulin	(Acid solution of pork and beef insulin).
Protamine Zinc Insulin (PZI)	(Neutral suspension of amorphous protamine-zinc-insulin complex, pork and beef insulin).
Ultralente	(Neutral suspension of beef insulin crystals).
Semilente	(Neutral suspension of pork insulin in the amorphous state).
Lente	(A mixture of seven parts Ultralente and three parts Semilente).
Rapitard	(Neutral suspension containing three parts beef insulin crystals and one part pork and beef insulin in solution).
Actrapid	(Neutral solution of pork insulin).

Ordinary Insulin and PZI were made of insulin crystallized at least twice, which had a biological activity of 25.0 U./mg. dry weight ± 2 per cent corresponding to 165 U./mg. N. The dry weight is determined after drying the insulin at 105° C. for eighteen hours. Ultralente, Semilente, Lente, Rapitard, and Actrapid were made of insulin crystallized at least three times, which had a biological activity of 26.1 U./mg. dry weight ± 2 per cent, corresponding to 171 U./mg. N. These figures of biological activity of the recrystallized insulin are the result of biological assays against the Fourth International Standard (mouse convulsion method) performed over five years. The stated potency of the insulin preparations under study was 40 U./ml. or 80 U./ml. calculated on the basis of the biological potency of the insulin used.

Biological insulin determinations were carried out on diluted solutions by the modified mouse convulsion method according to Young and Lewis, 1947.[10] For the preparation of the reference standards, use was made of the Fourth International Standard for insulin. The results were calculated by standard statistical methods for quantal response assays.[11]

Radioimmunological insulin determinations were carried out on diluted solutions by the ethanol precipitation method according to Heding, 1967.[12] For the preparation of the reference standards, use was made of the Fourth International Standard for insulin.

TABLE 1

Biological potency in per cent of the stated potency after storage for different periods at 4° C., 15° C. or 25° C. The figures outside the brackets show the experimentally found potencies with 95 per cent confidence limits, whereas the figures inside the brackets are derived from the formulas.

		0 yr.	1 yr.	2 yrs.	3 yrs.	4 yrs.	5 yrs.	8 yrs.	10 yrs.
4° C.	Ordinary insulin	99±2 (97)		94±5 (97)	97±5 (96)	98±16(96)	99±5 (96)		
	PZI	100±3(100)		97±7(100)	105±5(100)		94±5(100)		
	Ultralente	99±3 (96)		97±7 (96)	97±7 (96)		94±5 (96)		
	Semilente	101±2(100)		102±8(100)	102±6(100)		102±9(100)		
	Lente	99±1 (99)		105±9 (99)	106±6 (99)		103±9 (99)		
	Rapitard	100±2(100)		97±8(100)	102±7(100)		101±7 (99)		
	Actrapid	100±3(100)		96±8(100)	101±8(100)		98±6(100)		
15° C.	Ordinary insulin			91±4 (95)	90±4 (94)		90±5 (92)		
	PZI			100±5 (99)	95±5 (99)		97±6 (98)		
	Ultralente			98±8 (94)	88±5 (93)		85±6 (92)		
	Semilente			96±6 (99)	94±7 (99)		89±8 (98)		
	Lente			100±8 (98)	97±8 (97)		89±7 (96)		
	Rapitard		98±19(99)	92±7 (99)	98±6 (98)		98±6 (97)		
	Actrapid			104±8(100)	100±7 (99)		95±8 (98)		
25° C.	Ordinary insulin		95± 8(91)	84±3 (86)	84±3 (81)	69±10(76)	70±3 (71)	58± 8(60)	55±4 (53)
	PZI		105±11(96)	101±6 (92)	91±5 (88)		78±4 (82)	74±12(73)	70±6 (67)
	Ultralente			88±4 (85)	79±5 (80)	77± 8(75)	72±4 (70)	60± 6(58)	49±4 (51)
	Semilente			91±6 (94)	96±6 (91)	86±12(88)	84±3 (85)	76± 7(77)	76±6 (72)
	Lente		93±12(94)	86±7 (89)	87±7 (85)		76±5 (77)	64± 9(66)	62±5 (60)
	Rapitard		96±10(96)	96±7 (93)	87±7 (90)		88±7 (83)	73± 8(75)	
	Actrapid		103± 9(98)	99±7 (96)	94±6 (94)		92±7 (91)	84± 8(86)	

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/21/7/805/346849/21-7-805.pdf by guest on 30 March 2023

RESULTS

Bioassay

Tables 1 and 2 show the biological insulin activities expressed in per cent of the stated potency after storage of the insulin preparations in the dark for different periods of time at 4° C., 15° C., 25° C., 37° C. or 45° C. The figures outside the brackets show the experimentally found potencies with 95 per cent confidence limits, whereas the figures inside the brackets are derived from the formulas (9, 10, 11, 12, 13, 14, and 15). Since no difference has been found in the percentage loss in potency of the insulin preparations containing 40 U./ml. and those containing 80 U./ml., these results are pooled. Each experimental result in tables 1 and 2 is a weighed mean of results from two to twenty-six bioassays on different batches of the same type of insulin preparation. From 200 to 1,000 mice were used for each of these assays. A prerequisite for employing the mouse convulsion method for the measurement of the insulin potency is that the same dose of the unknown and the standard should have the same timing of action on the blood sugar of the mice. Measurements of the blood sugar both in mice and rabbits after injection of solutions of the insulin preparations in comparison with the Fourth International Standard showed that both freshly prepared insulin preparations and insulin preparations that had been stored for eight to ten years at 25° C. fulfilled this requirement.

Ultralente, Semilente and Lente have the same pH and the same concentration of $ZnCl_2$, NaCl, CH_3COONa, $3H_2O$ and methyl-p-hydroxybenzoate.[5] The only difference is that Ultralente contains beef insulin crystals, Semilente contains pork insulin in the amorphous state, while Lente is a mixture of seven parts Ultralente and three parts Semilente. Therefore, it would be natural to expect them to have the same stability. The results in tables 1 and 2 indicate, however, that Semilente is more stable than Ultralente at 25° C. By an analysis of variance a slight, but statistically significant difference ($P < 0.01$) is found between these preparations to the effect that Semilente is more stable and Ultralente less stable than Lente.

The biological potency ($P(t)$) of the insulin preparations as function of storage time (t) is assumed to follow a first-order reaction:

$$(1) \qquad P(t) = P_0 e^{-kt},$$

where P_0 is the initial potency, and k is the rate constant. A precise description of the dependence of the rate constant on the temperature, even over a wide temperature range, is obtained from the equation proposed by Arrhenius:[14]

TABLE 2

Biological potency in per cent of the stated potency after storage for different periods at 37° C. or 45° C. The figures outside the brackets show the experimentally found potencies with 95 per cent confidence limits, whereas the figures inside the brackets are derived from the formulas.

		1 mth.	2 mths.	4 mths.
37° C.	Ordinary Insulin		85± 8(90)	76± 8(84)
	PZI		95±10(89)	70± 6(80)
	Ultralente		78± 7(88)	79±11(81)
	Semilente		86±13(95)	87± 8(90)
	Lente		81± 7(92)	81± 6(85)
	Rapitard		93± 9(96)	88± 7(92)
	Actrapid		102±10(98)	94± 7(96)
45° C.	Ordinary Insulin	90±10(93)	75± 7(75)	
	PZI	76± 8(73)	54± 4(53)	
	Ultralente	84±10(83)	71± 5(71)	
	Semilente	90± 8(90)	83± 8(82)	
	Lente	87± 8(85)	74± 6(72)	
	Rapitard	89± 8(94)	91± 9(88)	
	Actrapid	98±11(97)	94± 8(94)	

$$(2) \qquad k = e^{(\alpha - \beta/T)},$$

where α and β are positive constants, and T is the absolute temperature. Accordingly, the formula for the potency as function of time and temperature is as follows:

$$(3) \qquad P(t,T) = P_0 e^{-te^{(\alpha-\beta/T)}}.$$

The experimentally found potencies are used to calculate the constants P_0, α and β for each of the seven types of insulin preparations. With the initial values $P_0 = P_0$, $\alpha = \alpha$ and $\beta = \beta$, an iterative method of regression analysis is set up to determine the parameter values.[13] Formula (3) is rewritten as equation (4):

$$(4) \qquad P(t,T) = (P_0 + \theta_1) e^{-te^{(\alpha + \theta_2 - (\beta + \theta_3)/T)}}.$$

θ_1, θ_2 and θ_3 are the deviations of the best fitting parameter values from the initial values P_0, α and β, respectively. (4) is considered as a function of the variables θ_1, θ_2 and θ_3 and is approximated around (0, 0, 0) according to Taylor.

$$(5) \qquad P(\theta_1,\theta_2,\theta_3) = P(0,0,0) + P'_{\theta_1}(0,0,0)\theta_1 + P'_{\theta_2}(0,0,0)\theta_2 + P'_{\theta_3}(0,0,0)\theta_3,$$

where

$$(6) \qquad P'_{\theta_1}(0,0,0) = e^{-te^{(\alpha-\beta/T)}},$$

$$(7) \qquad P'_{\theta_2}(0,0,0) = P_0(-te^{(\alpha-\beta/T)}) e^{-te^{(\alpha-\beta/T)}},$$

and

$$(8) \qquad P'_{\theta_3}(0,0,0) = P_0(-te^{(\alpha-\beta/T)})(-1/T) e^{-te^{(\alpha-\beta/T)}}$$

Using the observed relative potencies P_i, ($i = 1, 2, \ldots, N$), and the corresponding value t_i and T_i, the estimates θ_1, θ_2 and θ_3 are calculated according to the

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/21/7/805/348849/21-7-805.pdf by guest on 30 March 2023

TABLE 3

Calculated values for E_a (activation energy), $t_{0.5}$ (half-life) and Q_{10} (temperature coefficient)

	E_a cal./mol.	$t_{0.5}$ (yrs.) 5°C.	15°C.	25°C.	35°C.	45°C.	Q_{10} 5° → 15°C.	15° → 25°C.	25° → 35°C.	35° → 45°C.
Ordinary Insulin	30,300	450	67	11	2.2	0.46	6.7	5.9	5.3	4.7
PZI	42,900	3,200	210	17	1.7	0.18	14.8	12.3	10.5	9.0
Ultralente	31,800	510	70	11	1.9	0.38	7.3	6.4	5.7	5.1
Semilente	34,000	1,300	160	21	3.3	0.58	8.5	7.3	6.4	5.7
Lente	34,100	850	100	14	2.1	0.37	8.5	7.4	6.5	5.8
Rapitard	28,600	620	100	19	4.0	0.93	6.0	5.3	4.8	4.3
Actrapid	28,000	1,050	180	35	7.5	1.8	5.8	5.2	4.6	4.2

method of least squares by a linear multiple regression analysis. Then a new set of values for P_0, α and β: $P_0 + \theta_1$, $\alpha + \theta_2$ and $\beta + \theta_3$ are calculated. Using the new values for P_0, α and β in the analysis, new estimates for θ_1, θ_2 and θ_3 are calculated, and so on. Actually, weighed multiple linear regression analyses are performed at each step since the potency estimates have 95 per cent confidence intervals of different sizes. The square of the reciprocal half length of the confidence interval being proportional to the reciprocal of the variance of the potency estimates is used as weight factor. When $P_0 = 100$, $\alpha = 30$ and $\beta = 10000$ are used as initial values, the calculated values given in the seven formulas below become constant up to five significant digits within five steps of iteration. The seven calculated formulas are:

(9) Ordinary Insulin:

$$P_{OI} = 96.8\, e^{-te^{(45.9 - 15300/T)}}$$

(10) PZI:

$$P_{PZI} = 99.6\, e^{-te^{(66.6 - 21600/T)}}$$

(11) Ultralente:

$$P_U = 96.2\, e^{-te^{(48.4 - 16000/T)}}$$

(12) Semilente:

$$P_S = 99.9\, e^{-te^{(51.5 - 17100/T)}}$$

(13) Lente:

$$P_L = 99.0\, e^{-te^{(52.1 - 17200/T)}}$$

(14) Rapitard:

$$P_R = 99.8\, e^{-te^{(42.5 - 14400/T)}}$$

(15) Actrapid:

$$P_A = 100.3\, e^{-te^{(40.9 - 14100/T)}}$$

When t is recorded in months and T in °K, P will be expressed in per cent of the initial value. On the basis of the formulas, P can be calculated after storage under any time/temperature combination. In tables 1 and 2

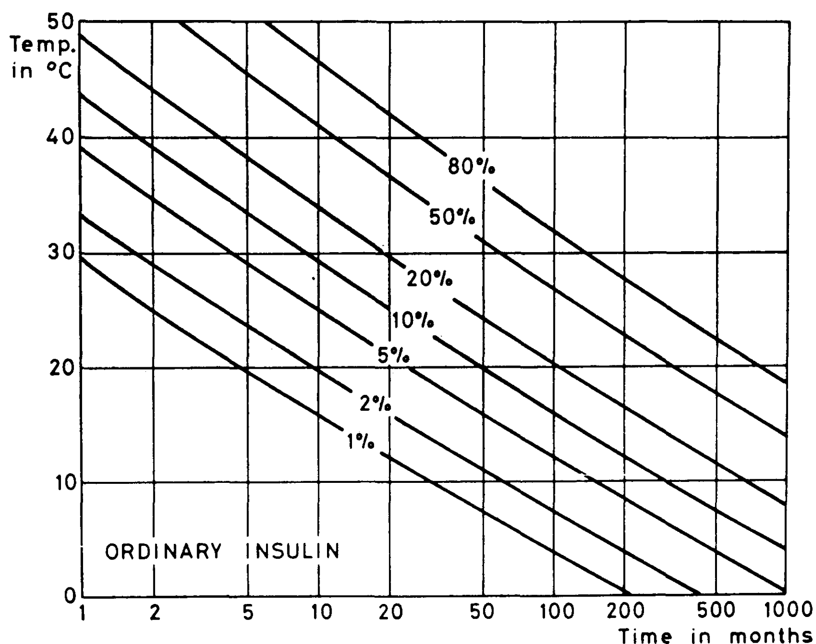

FIGURE 1

Loss of biological potency in per cent of the stated potency for Ordinary Insulin as a function of storage time and temperature.

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/21/7/805/348649/21-7-805.pdf by guest on 30 March 2023

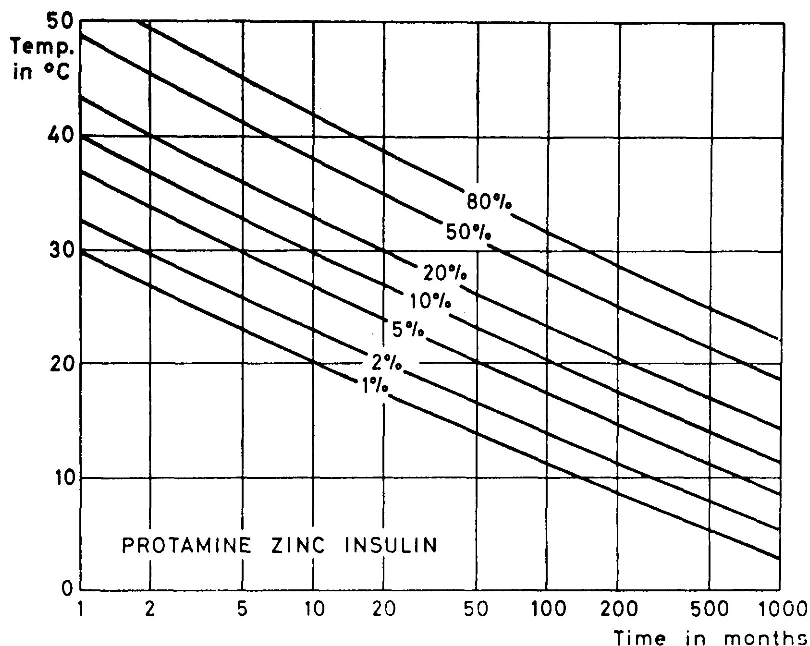

FIGURE 2

Loss of biological potency in per cent of the stated potency for PZI as a function of storage time and temperature.

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/21/7/805/346849/21-7-805.pdf by guest on 30 March 2023

the calculated biological potencies (figures in brackets) are shown for comparison with the measured potencies. With four exceptions, all the 120 calculated results agree with the 95 per cent confidence interval estimates for the experimental results.

The activation energy (E_a), the half-life ($t_{0.5}$) and the temperature coefficient (Q_{10}) can be calculated on the basis of the aforementioned values for α and β by means of the following equations:

$$(16) \quad E_a = R\beta,$$

$$(17) \quad t_{0.5} = \frac{\ln 2}{k} = \frac{\ln 2}{e^{(\alpha-\beta/T)}},$$

$$(18) \quad Q_{10} = \frac{K_{T+10}}{K_T} = \frac{e^{-\beta/(T+10)}}{e^{-\beta/T}},$$

where R is the gas constant. Table 3 shows the calculated values for E_a, $t_{0.5}$ and Q_{10} for each of the seven insulin preparations. E_a, or energy of activation, represents the energy a molecule must acquire before it can

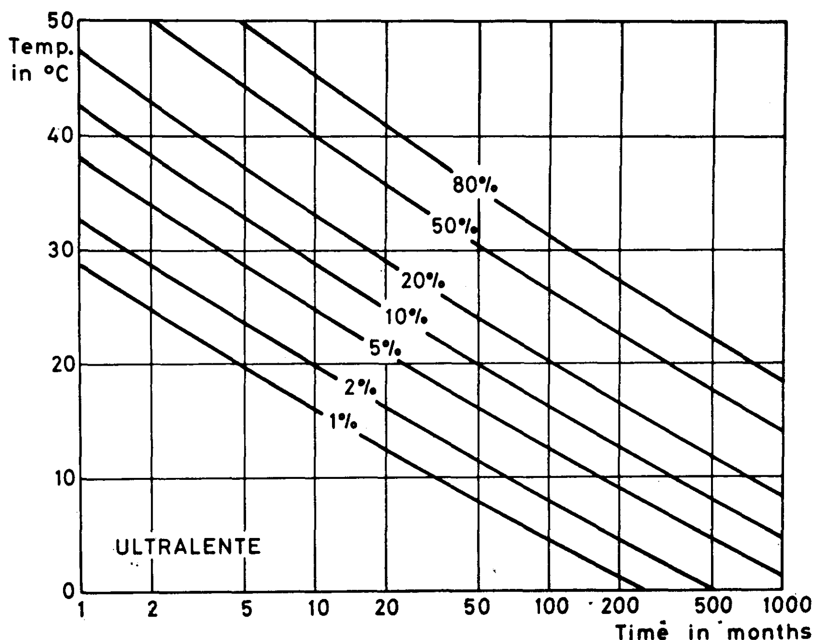

FIGURE 3

Loss of biological potency in per cent of the stated potency for Ultralente as a function of storage time and temperature.

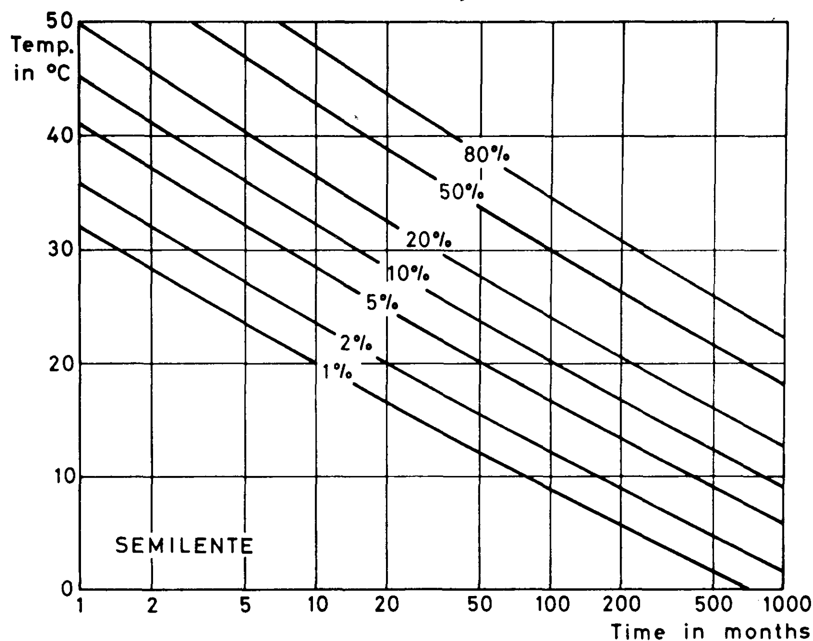

FIGURE 4

Loss of biological potency in per cent of the stated potency for Semilente as a function of storage time and temperature.

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/21/7/805/348849/21-7-805.pdf by guest on 30 March 2023

react.[14] The temperature coefficient Q_{10} represents the increase in the reaction rate when the temperature increases 10° C.[14] E_a is independent of the temperature, while $t_{0.5}$ and Q_{10} depend on the temperature. $t_{0.5}$ and Q_{10} are calculated at 5° C., 15° C., 25° C., 35° C. and 45° C. All the activation energies calculated are very high (28000-43000 cal./mol.), which then accounts for the marked influence of temperature on the reaction rate, since a rise in temperature will favor the formation of activated molecules.[14] The rate of disappearance of biological potency increases four- to fifteenfold with a temperature increase of 10° C.

Figures 1, 2, 3, 4, 5, 6 and 7 show the curves for 1, 2, 5, 10, 20, 50 and 80 per cent loss in biological potency as function of storage time and temperature calculated for Ordinary Insulin, PZI, Ultralente, Semilente, Lente,

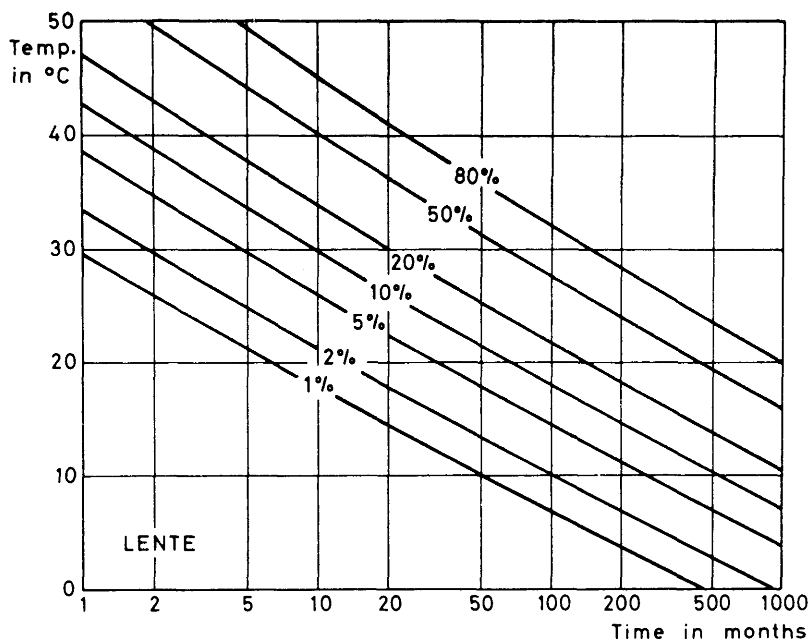

FIGURE 5

Loss of biological potency in per cent of the stated potency for Lente as a function of storage time and temperature.

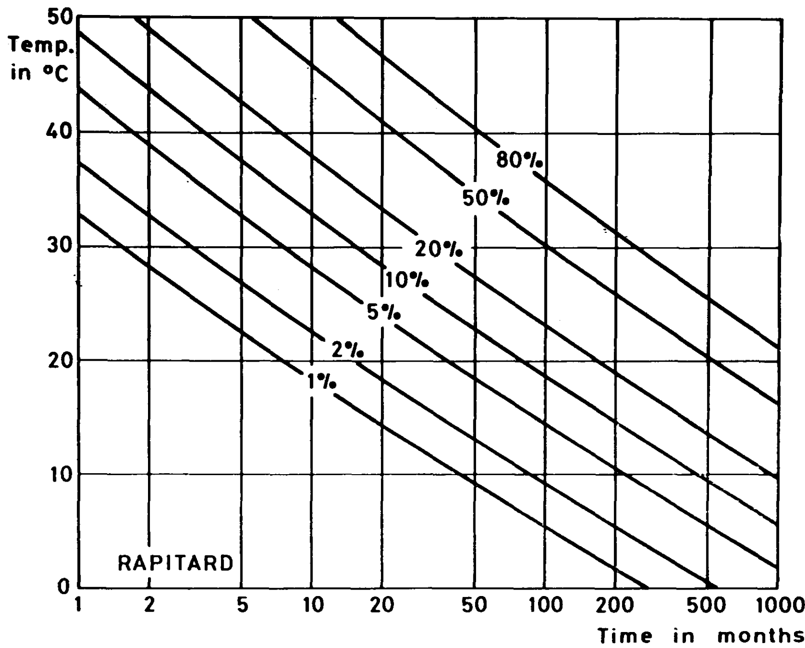

FIGURE 6

Loss of biological potency in per cent of the stated potency for Rapitard as a function of storage time and temperature.

Rapitard and Actrapid. These figures show that the loss in biological potency for all the insulin preparations is: Less than 1 per cent after storage for seven years at 5° C., one year at 15° C., two months at 25° C., nine days at 35° C., or one day at 45° C.; less than 5 per cent after storage for thirty-three years at 5° C., five years at 15° C., ten months at 25° C., forty-five days at 35° C., or five days at 45° C.; and less than 10 per cent after storage for sixty-nine years at 5° C., ten years at 15° C., twenty months at 25° C., three months at 35° C., or ten days at 45° C.

Radioimmunoassay

Tables 4 and 5 give the results of the immunological insulin determination of the insulin preparations after storage for different periods at 4° C., 15° C., 25° C., 37° C., or 45° C. in the dark, in per cent of the stated

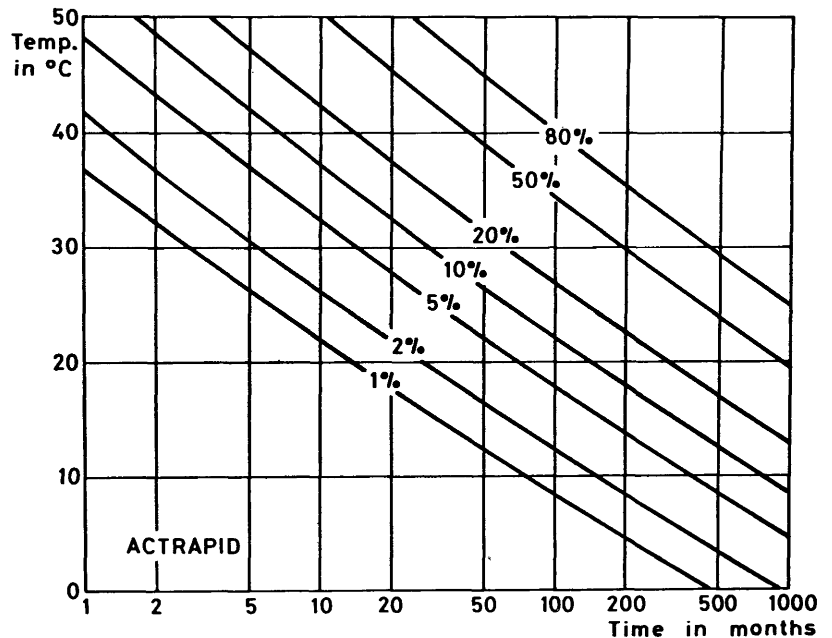

FIGURE 7

Loss of biological potency in per cent of the stated potency for Actrapid as a function of storage time and temperature.

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/21/7/805/348849/21-7-805.pdf by guest on 30 March 2023

TABLE 4

Immunological activity after storage at 4° C., 15° C. or 25° C., in per cent of the stated potency with 95 per cent confidence limits.

		3 yrs.	5 yrs.	8 yrs.	10 yrs.
4° C.	Ordinary Insulin	96±7	100±7		
	PZI	98±7	94±7		
	Lente	105±7	97±7		
	Rapitard	100±7	101±7		
	Actrapid	100±7	97±7		
15° C.	Ordinary Insulin	96±7	98±7		
	PZI	94±7	94±7		
	Lente	98±7	97±7		
	Rapitard	99±7	101±7		
	Actrapid	99±7	104±7		
25° C.	Ordinary Insulin	96±7	94±7	82±10	79±7
	PZI	88±7	84±7	68±10	72±7
	Lente	101±7	86±7	88±10	86±7
	Rapitard	97±7	92±7	86± 7	
	Actrapid	98±7	94±7	84± 7	

potency with 95 per cent confidence limits. Each result in tables 4 and 5 is a mean value of two immunoassays on two different batches of each of the insulin preparations.

Figure 8 shows a comparison between the biological and immunological insulin determinations expressed as the percentage loss of insulin activity with 95 per cent confidence limits. In fourteen cases the immunoassay shows insulin values 10 to 24 per cent higher than the bioassay. In thirteen of these cases (marked "S" in figure 8) the difference between the bioassay and the immunoassay is statistically significant ($P < 0.01$).

DISCUSSION

The close agreement between the values calculated from the formulas and the observed results, given in tables 1 and 2, is evidence for the validity of the assumptions on which the formulas were based. The assumption that the loss in biological potency follows a first-order reaction is also consistent with the results of Krogh and Hemmingsen,[1] Schlichtkrull[5] and Storvick and Henry.[7] The temperature dependence of the rate constant, see equation (2), was analyzed by Krogh and Hemmingsen[1] for acid insulin solutions at 50° C. to 117° C. From their results we calculated $\alpha = 36$ and $\beta = 14{,}200$. The value for β is of the same order as ours. The results for the decrease in biological insulin activity found by Schlichtkrull[5] agree with our values. In agreement with Storvick and Henry[7] we found all neutral insulin preparations more stable than acid insulin preparations. The results of the immunoassay reported by Storvick and Henry[7] at low temperatures ($T \leqq 15°$ C.) are consistent with the results of their

TABLE 5

Immunological activity after storage at 37° C. or 45° C., in per cent of the stated potency with 95 per cent confidence limits.

	2 mths.—45° C.	4 mths.—37° C.
Ordinary Insulin	93±7	91±7
PZI	51±7	67±7
Lente	91±7	92±7
Rapitard	94±7	92±7
Actrapid	90±7	89±7

bioassay, our immunoassay and our bioassay. Contrary to this, at higher temperatures ($T \geqq 25°$ C.), the results of our bioassay are inconsistent both with our own immunoassay and with their immunoassay. For instance, we found in thirteen cases that the immunoassay showed insulin values 10 to 24 per cent higher than the bioassay ($P < 0.01$). This means that, contrary to Storvick and Henry,[7] we can draw the conclusion that the immunoassay cannot replace the bioassay for unknown insulin preparations. The reason for the divergency between Storvick and Henry's[7] and our conclusion appears to be that they performed the comparison of the two assays on preparations stored at 5° C. only.

From figure 8 it appears that whenever the loss in biological potency exceeded 20 per cent it was markedly higher than the corresponding loss in immunological potency except for Protamine Zinc Insulin which showed no appreciable difference. In the case of Ordinary Insulin stored at 25° C. for ten years, the immunological potency was found to be 40 per cent higher than the biological potency, while the corresponding difference for PZI was only 3 per cent.

Although all the insulin preparations tested exhibit a remarkably high stability of biological potency, there may be other factors of therapeutical significance which could change more readily during storage. The stability with respect to the timing of action has been less thoroughly investigated. However, Schlichtkrull[5] found no change in the timing of blood sugar lowering effect of the Lente insulins when tested in rabbits after two years of storage at 20 to 25° C.

ACKNOWLEDGMENT

We are grateful to J. Schlichtkrull, D.Sc., and to H. Ege, M.Sc., for their interest and suggestions during the work and the preparation of the manuscript. We also wish to thank L. G. Heding, M.Sc., Lic. Techn., and E. Parbst, M.Sc., for carrying out the immunoassays, as well as E. Sørensen, M.Sc., and P. Nielsen, M.Sc., for carrying out the bioassays.

REFERENCES

[1] Krogh, A., and Hemmingsen, A. M.: CLIII. The destructive action of heat on insulin solutions. Biochem. J. *XXII*:1231, 1928.

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/21/7/805/346849/21-7-805.pdf by guest on 30 March 2023

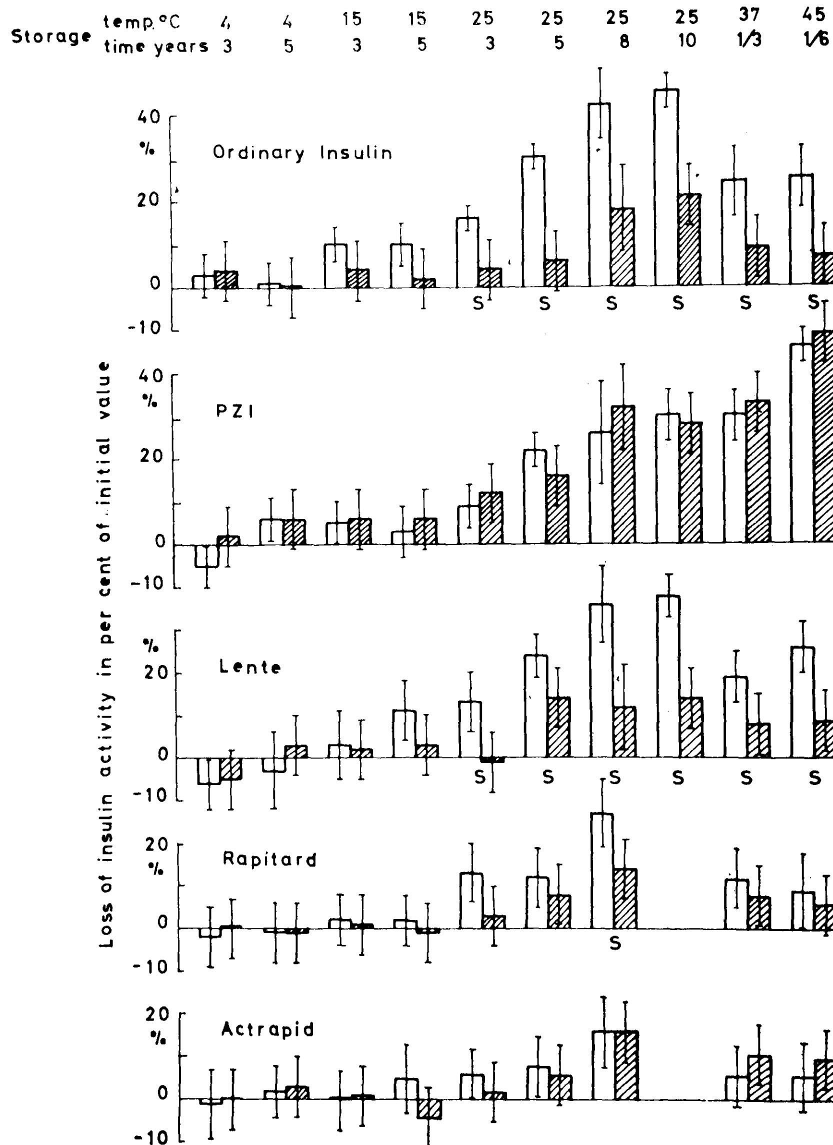

FIGURE 8

Loss of insulin activity of different insulin preparations after storage, in per cent of the stated potency with 95 per cent confidence limits, determined by bioassay (nonhatched columns) and by immunoassay (hatched columns).

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/21/7/805/346849/21-7-805.pdf by guest on 30 March 2023

[2] Sahyun, M., Goodell, M., and Nixon, A.: Factors influencing the stability of insulin. J. Biol. Chem. *117*:685, 1937.

[3] Sahyun, M., Nixon, A., and Goodell, M.: Influence of certain metals on the stability of insulin. J. Pharmacol. Exp. Ther. 65:143, 1939.

[4] Lens, J.: The inactivation of insulin solutions. J. Biol. Chem. *169*:313, 1947.

[5] Schlichtkrull, J.: Insulin crystals. Chemical and biological studies on insulin crystals and insulin zinc suspensions. Diss.: Copenhagen University 1958, p. 96.

[6] Stephenson, N. R., and Romans, R. G.: Thermal stability insulin made from zinc insulin crystals. J. Pharm. Pharmacol. *XII*:372, 1960.

[7] Storvick, W. O., and Henry, H. J.: Effect of storage temperature on stability of commercial insulin preparations. Diabetes *17*:499, 1968.

[8] Peck, F. B., and Schechter, J. S.: The newer insulin mixtures. Proc. Amer. Diabetes Ass. *4*:57, 1944.

[9] Rückert, A., and Schöne, J.: Uber die Inaktivierung von Insulin durch Sulfat-Ionen. Die Pharmazie *16*:545, 1962.

[10] Young, D. M., and Lewis, A. H.: Detection of hypoglycemic reaction in the mouse assay for insulin. Science *105*: 368, 1947.

[11] BP: British Pharmacopoeia 1968, p. 1309.

[12] Heding, L.: A simplified insulin radioimmunoassay method. *In* Labelled Proteins in Tracer Studies. Brussels, 1966, p. 345-50.

[13] Williams, E. J.: Regression analysis. New York, John Wiley and Sons, Inc., 1959, p. 59-71.

[14] Glasstone, S.: Textbook of Physical Chemistry, Second Edition. New York, D. van Nostrand Company, Inc., 1946, p. 1087-91.