TABLE I. Statistical Analysis and Summary of Results.

Condition	Control	x	s	n	x *vs* 100%		x *vs* N_2 8-10 atm.	
					t	p	t	p
1. O_2 8 atm.	.143 ± .086	65.4	10.6	11	10.9	.001	13.7	<.001
2. O_2 8, 10, 12 "	.153 ± .076	57.4	10.9	15	15.1	.001	10.9	"
3. N_2 8, 10 "	.134 ± .050	111.5	17.8	7	1.7	0.15		

The means, x, are in % of 1 atm. air control taken as 100%.

centage of that in its paired control. The means (x) of these values (Table I) and their standard deviations (s) were found when (1) oxygen pressure equals 8 atmospheres, (2) oxygen pressure equals 8, 10, and 12 atmospheres, and (3) nitrogen pressure equals 8 or 10 atmospheres. The means (x) were first compared to 100% (the air control); then the high oxygen experimental means were compared to the nitrogen experimental mean in terms of t-value (t) and probability (p). Number of experiments in each series is denoted by (n). In all cases the formula(5)

$$t = \frac{x_1 - x_2}{Sp\sqrt{\frac{1}{n_1} + \frac{1}{n_2}}}$$

was used where Sp is the standard deviation of the pool.

There was a great variation in absolute value of the transport of sodium in different experiments. The average control with standard deviation is expressed in Table I in microequivalents of sodium per centimeter square per hour (μ eq/cm^2/hr.).

In conditions 1 and 2, mean transport in the high oxygen tension is significantly lower than the control values (1 atm. air) but in condition 3, the high nitrogen pressure without deprivation of oxygen had no significant effect. Furthermore, comparison of the effects of high oxygen tension and of high nitrogen tensions showed that mean transport was significantly lower in the former case.

Therefore, we may conclude that oxygen tensions in excess of 8 atmospheres markedly inhibit sodium transport in the frog skin and that high pressures of nitrogen with normal oxygen tensions (*i.e.*, high pressures *per se*) do not have this inhibitory effect. These observations are consistent with the idea that excess of O_2 interferes with the normal activity of oxidative enzymes and inhibits sodium transport by diminishing the supply of energy available for the purpose.

Summary. Oxygen at 8 to 12 atmospheres depresses the transport of sodium ion across the isolated frog skin to 57.4%. Addition of 8 to 10 atmospheres of nitrogen without oxygen deprivation had no such effect.

1. Huf, E., Wills, J., Cooley, M. J., *Arch. f.d. ges. Physiol.*, 1952, v255, 16.

2. Ussing, H. H., Zerahn, K., *Acta Physiol. Scand.*, 1951, v23, 110.

3. Cass, R. E., *Am. J. Physiol.*, 1947, v148, 490.

4. Gerschman, R., Gilbert, D. L., Nye, S. W., Dwyer, P., Fenn, W. O., *Science*, 1954, v119, 623.

5. Dixon, W. J., Massey, F. J., *Introduction to Statistical Analysis*, McGraw-Hill, 1957.

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Rapid Production and Detection of Insulin-Binding Antibodies in Rabbits and Guinea Pigs. (25074)

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Insulin has generally been regarded as weakly antigenic in man and animals(1,2,3). Yet it has been demonstrated that virtually all humans receiving insulin therapy for more than a few weeks develop circulating insulin-binding antibodies demonstrable by technics employing I^{131}-labeled insulin and paper elec-

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trophoresis(4). Although Arquilla and Stavitsky were able to immunize rabbits with alum-precipitated crystalline insulin(5), Lowell and Franklin(6) and Moloney and Coval(7) found it difficult to immunize rabbits with unmodified insulin in Freund adjuvants, whereas guinea pigs responded uniformly with antibody production(7). The present study reports uniform development of insulin-binding antibodies in giunea pigs and rabbits immunized with unmodified crystalline beef or crystalline pork insulin in a modified Freund adjuvant.

Methods. Crystalline beef or pork insulin§ in physiological saline (1 mg/ml) was homogenized at high speed in a Virtis Homogenizer for 1 minute with equal volume of adjuvant (⅔ mineral oil-Bayol F.†; ⅓ Aquaphor‡). Five rabbits (3-5 kg) were injected subcutaneously with .5 mg insulin 3-5 times/week for 1½ to 9 weeks. Seventeen guinea pigs (300-710 g) received one subcutaneous injection 0.1-0.25 mg insulin and occasionally a second injection 3-6 weeks later. Despite apparently complete emulsification of the insulin-adjuvant homogenate, 3 guinea pigs had fatal hypoglycemic reactions. Crystalline insulins were labeled with I^{131} by methods described(4) yielding specific activity of 3-24 mc I^{131}/mg insulin and average less than 1 iodine atom/molecule 12,000 M.W. insulin. Disappearance of radioactivity following subcutaneous injection of insulin-I^{131} with and without adjuvant was measured by gamma ray scintillation counter. Times for absorption of 50% of insulin-I^{131} were 1¼, and 2 hours without adjuvant and 4 and 12 hours with adjuvant for 2 experiments in each group (Fig. 1). The extent of binding of insulin by antibody was determined by paper electrophoresis or hydrodynamic flow paper chromatography in veronal buffer pH 8.6, employing insulin-I^{131}(4). When plasma or serum containing insulin-I^{131} is placed on Whatman 3 MM paper, unbound insulin is adsorbed to the paper

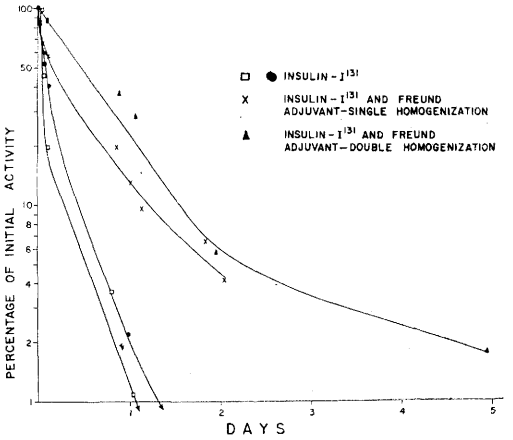

FIG. 1. Disappearance from subcut. inj. sites of crystalline beef insulin-I^{131} with and without modified Freund adjuvant.

at site of application ("origin"), whereas insulin bound to antibody migrates away from the origin with the binding protein(4). Homogenization with adjuvant produced no alterations in insulin-I^{131} detectable by paper electrophoresis, employing methods described elsewhere for detection of damage to I^{131} labeled proteins(4,8). All sera were incubated with the antigen for 4 hours at 37°C to allow maximum binding by the antibody.

Results. Insulin antibodies were demonstrable in all 5 rabbits within 11-36 days, and in all 14 surviving guinea pigs within 10-63 days after initial injection. On electrophoresis, insulin-antibody complexes in rabbit plasma migrated with the γ globulins (Fig. 2) and, in guinea pig plasma, in region of fibrinogen. Insulin-antibody complexes in human antiserum migrate in the inter β-γ zone(4). Normal guinea pigs, normal rabbits and rabbits immunized with human serum albumin or with rheumatoid arthritis factor showed no plasma binding of labeled insulin.

When insulin concentration is varied but antiserum concentration kept constant, the percentage of bound insulin decreases progressively with increase in insulin concentration, whereas the absolute quantity of bound insulin increases to an asymptotic value(4) which represents maximum binding capacity (Fig. 3). Maximum insulin-binding capacities ranged from less than 10 to 5000 mμg insulin/ml antisera in guinea pigs and from

§ We are indebted to Dr. O. K. Berens and Dr. C W. Pettinga of Eli Lilly Co., Indianapolis, for generous gifts of crystalline beef and pork insulin.

† Bayol F. Imperial Oil Co.

‡ Aquaphor Duke Lab., Norwalk, Conn.

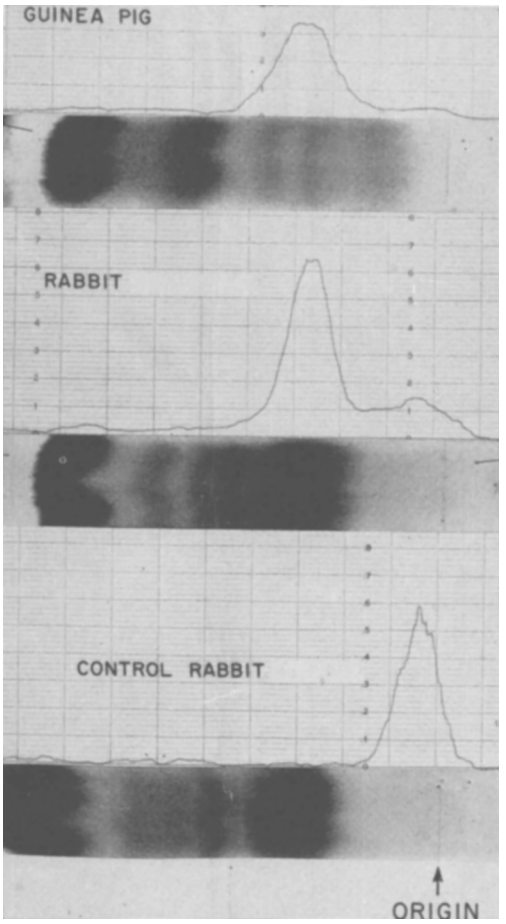

FIG. 2. Paper radioelectrophoretograms comparing migration of beef insulin-I^{131} in plasma of immunized guinea pigs (top) and rabbits (middle) and control rabbits (bottom).

14-550 mμg/ml in rabbits. Even much lower concentrations of antibody are detectable by these technics.

No precipitation was observed after 1 week of refrigeration in mixtures in which concentrations of bound insulin were 1/10th the maximum binding capacity of the antiserum.

Discussion. Successful demonstration of insulin-binding antibodies following immunizing procedure described here depends, first, upon use of a well emulsified, semi-solid preparation which markedly retards the otherwise rapid absorption of the small antigen from injection site, and secondly upon the very high sensitivity for detection of binding by antibody. Previous assays of antibody activity have included: resistance to insulin induced hypoglycemia in immunized rabbits(6), passive transfer of resistance in mice(7), systemic anaphylaxis in the sensitized guinea pig(9,10), complement fixation in rabbits(11), and agglutination of rabbit antisera with insulin conjugated rabbit and sheep erythrocytes(5). Sensitivity of these methods varies considerably and it is possible that the lowest titers of antibody noted here might well have gone undetected by other methods. Failure to demonstrate precipitating antigen-antibody complexes is in agreement with other authors(5) although concentrations of antigen-antibody complexes were frequently sufficiently low that, on this account alone, precipitation might not have occurred.

The effect of insulin-binding antibodies on response to hormonal action of insulin is currently under study. However, it must be emphasized that in most immunized animals total insulin-binding capacities were so low that significant insulin resistance is not to be anticipated.

Summary. Rabbits and guinea pigs were immunized with untreated crystalline beef and pork insulin in modified Freund adjuvant. Disappearance of insulin-I^{131} from subcutaneous sites of injection was markedly reduced by the adjuvant. Fourteen of 17 guinea pigs and all 5 rabbits survived otherwise lethal doses of insulin. Insulin-binding antibodies were produced within 11-36 days in all rabbits and within 10-63 days in all surviving guinea pigs. Insulin-antibody complexes in rabbit plasma migrate with γ globulins and in guinea pig plasma with fibrinogen,

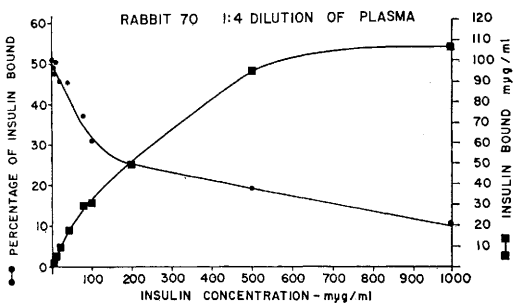

FIG. 3. Quantity of beef insulin-I^{131} bound (■) and percentage of beef insulin-I^{131} bound (●) in rabbit anti-beef insulin serum as a function of unlabeled beef insulin concentration.

on paper electrophoresis. Total insulin-binding capacities of antisera ranged from less than 10 to 5000 mμg/ml in guinea pigs and from 14 to 550 mμg/ml serum in rabbits.

1. Lerman, J., *Am. J. Med. Sci.,* 1944, v207, 354.
2. Heidelberger, M., *Lectures in Immunochemistry,* 1956, p17, Acad. Press, N. Y.
3. Haurowitz, F., *Chemistry and Biology of Proteins,* 1950, p284, Acad. Press, N. Y.
4. Berson, S. A., Yalow, R. S., Bauman, A., Rothschild, M. A., Newerly, K., *J. Clin. Invest.,* 1956, v35, 170.
5. Arquilla, E. R., Stavitsky, A. B., *ibid.,* 1956, v35, 458.
6. Lowell, F. C., Franklin, W., *ibid.,* 1949, v28, 199.
7. Moloney, P. J., Coval, M., *Biochem. J.,* 1955, v59, 179.
8. Berson, S. A., Yalow, R. S., *Ann. N. Y. Acad. Sci.,* 1957, v70, 56.
9. Bernstein, C., Kissner, J. B., Turner, W. J., *J. Lab. and Clin. Med.,* 1938, v23, 938.
10. Wasserman, P., Mirsky, I. A., *Endocrin.,* 1942, v31, 115.
11. Wasserman, P., Broh-Kahn, R. H., Mirsky, I. A., *J. Immunol.,* 1940, v38, 213.

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Hyperlipemia and Hemolysis III. Acceleration of Oleate Lysis of Human Erythrocytes by Homologous Plasma. (25075)

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In a previous study(1), human erythrocytes were found to compete *in vitro* with homologous plasma for binding exogenous sodium oleate. That portion of oleate bound by plasma was rendered hemolytically inert; even vigorous mechanical agitation failed to dissociate the binding appreciably. That portion of exogenous oleate bound by the erythrocyte constituted the active hemolytic moiety. The present report concerns the nature and mechanism of human plasma acceleration of hemolysis following erythrocyte-oleate binding; these findings extend earlier reports(2) indicating that once human erythrocytes bind oleate, homologous plasma reverses its hemolytic protective action and accelerates oleate lysis. Such studies are a continuation of investigations regarding possible intermediate pathways for *in vivo* hemolysis observed during conditions associated with rapid increases in plasma unesterified fatty acid(3-6). From our present findings, a scheme is inferred which may clarify certain events linking *in vivo* plasma unesterified fatty acid increases with subsequent hemolysis.

Materials and methods. Heparinized blood of all major groups was collected from normal human subjects, spun at 2500 rpm, 0°C, 15 minutes, and erythrocytes washed 3 times in sodium phosphate buffer, pH 7.2, ionic strength 0.15. Unless otherwise specified, standardized erythrocyte preparations were employed wherein addition of 0.5 ml of erythrocyte suspension to 2.5 ml of fatty acid or control solutions resulted in final concentration of 5 x 10^5 cells/cu mm. All fatty acid solutions were prepared in sodium phosphate buffer. The effect of temperature on pH of such buffered sodium oleate (Baker) solutions, measured with glass electrode, was as follows: 37°C, pH = 7.10; 25°C, pH = 7.20; 0°C, pH = 7.30. All fatty acid solutions were allowed to remain at room temperature at least 24 hours prior to use, since the hemolytic potency declined upon standing, rapidly at first, then more slowly. Human plasma albumin and globulin fractions were prepared from 3 individual citrated normal plasma specimens by overnight dialysis against ammonium sulfate solutions at 4°C employing magnetic stirring. Globulin-rich fractions were obtained at 50% ammonium sulfate saturation; albumin-rich fractions at 100% saturation. The fractions were washed with appropriate ammonium sulfate solutions, resuspended in buffered isotonic saline (pH 7.4) and cleared of ammonium ion (verified with Nessler's reagent) by dialysis at 4°C