LIPOPROTEIN TRANSFORMATIONS UNDER THE INFLUENCE OF HEPARIN AND *CLOSTRIDIUM WELCHII* α-TOXIN

by

G. R. WILLIAMS*

Banting and Best Department of Medical Research, University of Toronto, Toronto, Ont. (Canada)

Heparin administered intravenously abolishes post-absorptive lipaemia (HAHN[1], WELD[2]). The addition of heparin to lipaemic plasma *in vitro* does not affect the turbidity but ANDERSON AND FAWCETT[3] have shown that the plasma of heparinized dogs is active in this respect, *i.e.* that the addition of such plasma to lipaemic plasma is followed by a reduction in turbidity. ANFINSEN, BOYLE AND BROWN[4] have demonstrated that this lipaemia clearing activity can be concentrated by alcohol fractionation of heparinized plasma. SPITZER[5] has also concentrated the "clearing factor" by ammonium sulphate fractionation and there appears to be a discrepancy between his results and those of ANFINSEN *et al.* but differences in the fractional precipitation methods employed may account for the fact that these two groups of workers find the clearing activity to be associated with very different protein fractions.

The mechanism of this effect has been elucidated by the ultra-centrifugal studies of GRAHAM, LYON, GOFMAN, JONES, YANKLEY, SIMONTON AND WHITE[6], and of BOYLE, BRAGDON AND BROWN[7], who have shown that the dissolution of the chylomicra noted by SWANK AND WILMOT[8] and SWANK AND LEVY[9] is accompanied by the production of low-density lipoproteins which are in turn converted to lipoproteins of lower flotation rates. These products may presumably be identified with the β- and α-lipoproteins isolated by electrophoresis (KUNKEL AND SLATER[10]) or by chemical fractionation (ONCLEY, SCATCHARD AND BROWN[11], RUSS, EDER AND BARR[12]) and we may therefore summarize the clearing activity of the chylolytic material formed on intravenous injection of heparin thus, where CF (clearing factor) designates the active material

$$\text{Chylomicra} \xrightarrow{CF} \beta\text{-Lipoproteins} \xrightarrow{CF} \alpha\text{-Lipoproteins}$$

Such a scheme receives strong support from the paper electrophoretic studies of NIKKILA[13].

This communication reports the results of experiments carried out to cast further light upon the kinetics and mechanisms of these interesting transformations.

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EXPERIMENTAL

Materials and Apparatus

Certain preliminary experiments were performed using plasma from heparinized rats without fractionation, but the majority of the experiments reported here were performed using a globulin fraction prepared from plasma withdrawn from a dog ten minutes after intravenous injection of 50,000 i.u. of heparin (Connaught, Toronto). The fractionation was carried out essentially according to methods 6 and 9 of COHN and his associates[14,15] and following ANFINSEN *et al.*[4] fraction III, 1, 2, 3 was isolated as a source of clearing factor. Two such preparations were utilized, no significant difference being found between the two lots.

A preparation of *Clostridium welchii* alpha-toxin was used as a source of phospholipase C. This toxin (lot 024354) was kindly supplied by Dr. G. D. BRIGHAM of Parke, Davis & Co., Detroit, U.S.A. and had an MLD value of 0.1 ml given intraperitoneally and 0.06 ml by intravenous route in 16–20 g Swiss mice.

Lipaemic serum was obtained from dogs 2–3 hours after giving a fatty meal. 0.15 *M* NaCl solution in deionized water was used as solvent for the lyophilized clearing factor preparation and as diluent for lipaemic sera.

Turbidities were measured by transmission measurements at 700 mμ, all measurements being made in a spectrophotometer employing a Fery prism as monochromator (Beckman Model "B").

RESULTS

Kinetics

It is unlikely that the decrease in turbidity which occurs on incubation of lipaemic sera with clearing factor at room temperature (Fig. 1) will obey any equation corresponding to a simple order of reaction. This unlikelihood is increased by the fact that the dissolution of the chylomicra may pass through intermediate stages corresponding to particles which would themselves scatter light. While these expectations are, in fact, realized certain features do emerge during experimental study and a number of statements may be made regarding the progress of the transformation.

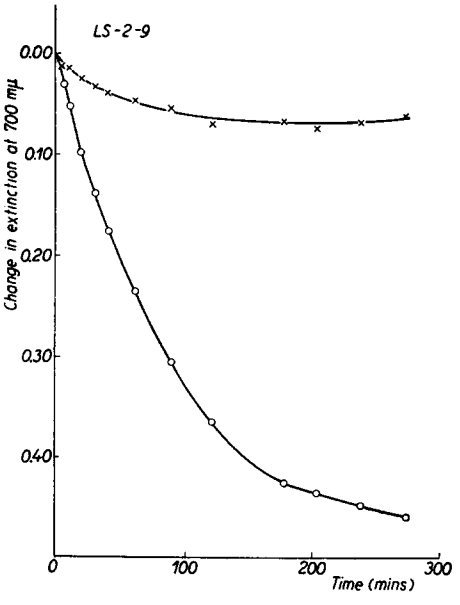

Fig. 1. The decrease in turbidity of lipaemic serum on incubation at 23° C with clearing factor.
–O–O–O– 1 ml lipaemic serum (dog), 1 ml of clearing factor solution, 1 ml of saline.
–×–×–×– 1 ml lipaemic serum (dog), 1 ml normal human serum, 1 ml saline.

Firstly, the reaction does not proceed to completion *in vitro* even at high concentrations of clearing factor. After a period of time which varies from one and a half to two hours the fall in turbidity stops. The absolute fall in extinction varies with the initial turbidity but if this change is expressed as a fraction of the initial turbidity a constant ratio, $\frac{T_o - T_{eq}}{T_o}$ is obtained, where T_o is the initial turbidity and T_{eq} the final extinction value. We have designated this ratio the "fractional clearing" F.C. This is shown in Table I and Fig. 2.

In this table the initial turbidities, T_o are corrected for the turbidity of the clearing factor which is not negligible. It has proved impossible in these and numerous other experiments to show any dependence of the initial rate of clearing on the turbidity at zero time such as

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TABLE I

EXTENT OF CLEARING OF LIPAEMIC SERUM (DOG) BY CONCENTRATED CLEARING FACTOR. T_0 = INITIAL TURBIDITY; T_{eq} = FINAL TURBIDITY REACHED. CONCENTRATION OF LYOPHILIZED GLOBULIN FRACTION PREPARED FROM HEPARINIZED PLASMA (dog) BY LOW TEMPERATURE ALCOHOL FRACTIONAL PRECIPITATION = 0.33 % (w/v)

T_0	T_0-T_{eq}	*Fractional clearing* $\frac{T_0-T_{eq}}{T_0}$
0.095	0.069	0.73
0.180	0.129	0.72
0.260	0.204	0.78
0.280	0.219	0.78
0.339	0.250	0.74
0.420	0.313	0.74
0.423	0.330	0.78
0.482	0.365	0.76
0.555	0.406	0.73

would be expected if the system obeyed the MICHAELIS-MENTEN formulation for an enzyme catalyzed reaction (*cf.* Fig. 2). If such a formulation holds, the K_m of the system must correspond to a low extinction value and, using the Beckman Model B instrument it is difficult to make accurate measurements of the change in turbidity when the initial value of the extinction/cm. falls much below 0.100. It is hoped further to examine this problem using a sensitive nephelometer which has been constructed.

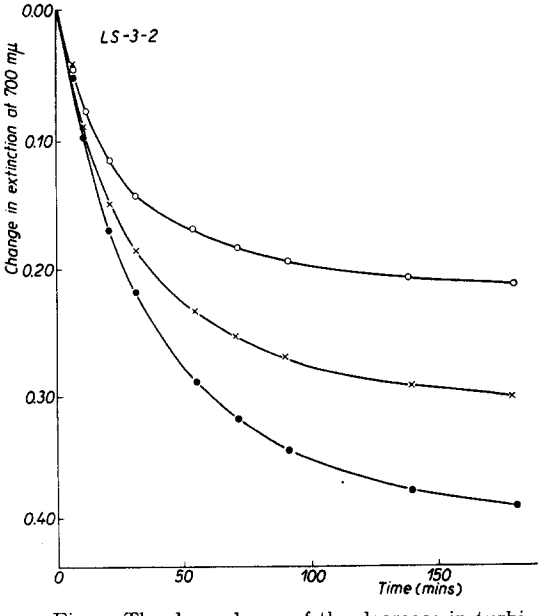

Fig. 2. The dependence of the decrease in turbidity upon the initial turbidity. 0.2, 0.3, 0.4 ml of lipaemic serum (dog) were respectively added to 1 ml of clearing factor solution (1 % wt/v) and the vol. made up to 3 ml with saline.
-O-O-O- initial turbidity = 0.280
-×-×-×- initial turbidity = 0.420
-●-●-●- initial turbidity = 0.555

It follows from the above that the information given by ANFINSEN *et al.*[4] regarding the activities of their various plasma fractions (i.e. change in optical density/hour/mg protein) is strictly speaking insufficient for complete characterization as the amount of clearing which occurs is a function of the initial turbidity. However, one agrees with their finding of a relationship between the concentration of clearing factor and the degree of clearing (Fig. 3).

One further relationship has been found. In a high proportion of the progress curves examined (13 out of 16) there appears to be for the first forty minutes a straight line relationship between the inverse of the turbidity and time elapsed since the start of the reaction, *i.e.* $\frac{1}{t} \cdot \frac{T_o - T_t}{T_o \cdot T_t}$ is a constant (Table II) over the initial period of clearing.

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The constant obtained defines any given progress curve but cannot be regarded as a velocity constant as it varies with the initial concentration. The formal resemblance to the equation for a second order reaction is therefore coincidental as indeed might have been expected from a consideration of the nature of the system, it being unlikely that the interaction of two chylomicra could form a basis for the phenomenon under discussion. It is however possible that this "constant" may prove useful in the comparison of clearing reactions in different systems, *e.g.*, with sera of different origins as "substrate".

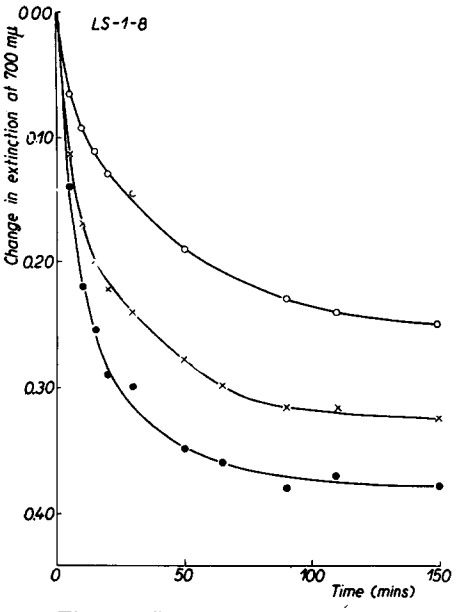

Fig. 3. Effect on the decrease in turbidity of varying the amount of clearing factor (unfractionated heparinized rat plasma).
-O-O-O- 0.5 ml of clearing factor preparation
-×-×-×- 1.0 ml of clearing factor preparation
-●-●-●- 1.5 ml of clearing factor preparation

TABLE II

RATE OF CLEARING OF LIPAEMIC SERUM (dog): t = TIME (IN MINS) T_0 = INITIAL TURBIDITY, T_t = TURBIDITY AT TIME t

t	T_t	$k = \frac{1}{t} \cdot \frac{T_0 - T_t}{T_0 \cdot T_t}$
0	0.423	
2	0.402	0.0650
5	0.371	0.0642
10	0.333	0.0639
18	0.281	0.0664
23	0.253	0.0690
30	0.233	0.0643
40	0.203	0.0641

Interaction with the NAGLER *effect*

NAGLER[16] first showed that on culturing *Clostridium welchii* Type A in human serum an opalescence appeared due to the liberation of fat from lipid-protein complexes. This effect which can be observed only in human serum and plasma has subsequently been investigated by a number of workers[17, 18, 19, 20] and it has been shown that the instability of the native lipoproteins and their consequent breakdown is brought about by the hydrolysis of phospholipids by an enzyme present in the α-toxin, these phospholipids being presumably essential in some way for lipoprotein stability. This effect is, at least superficially, a reversal of the effect of clearing factor but no investigation appears to have been made of their interrelationships. It appeared that such an investigation might cast light on both reactions. We have therefore examined the effect of clearing factor on dog lipaemic sera after pre-incubation with *Cl. welchii* toxin and the effect of *Cl. welchii* toxin on human sera pre-incubated with clearing factor.

In the first case a clear cut effect is obtained. If lipaemic dog serum is incubated with *Cl. welchii* toxin for a short time it becomes impossible to produce a fall in turbidity on subsequent treatment with clearing factor (Fig. 4).

The second case does not yield such a clear-cut result. Nineteen human sera have been examined. Ten were taken from young adults in good health working in this

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Department, seven from a group of patients with diabetes and/or liver disease[21], and two from a group of patients with atherosclerosis. In the first group nine of the ten sera

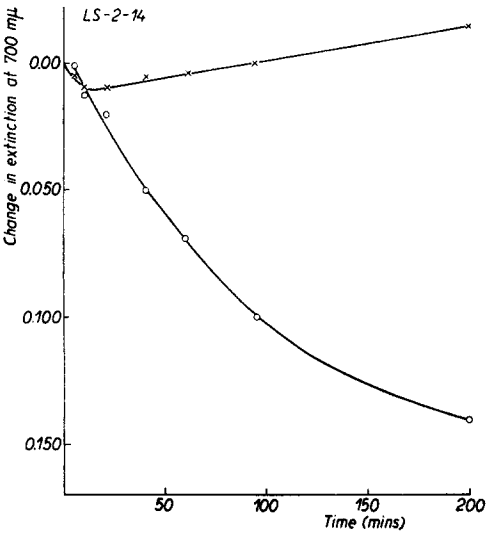

Fig. 4. Failure to elicit the clearing response after pre-treatment with *Cl. welchii* α-toxin.
-O-O-O- prior incubation with saline
-×-×-×- prior incubation with toxin.

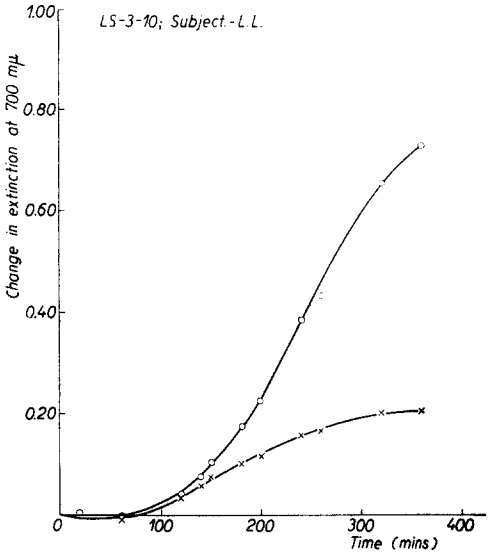

Fig. 5. The suppression of the NAGLER effect by pre-incubation with clearing factor.
-O-O-O- saline control
-×-×-×- pre-treated with clearing factor.

showed a suppression of the NAGLER effect by pre-incubation with clearing factor. This effect is shown in Fig. 5, but not all sera showed such a complete resistance to the toxin as the case presented; however in all nine cases the increase in turbidity brought about by the toxin was diminished to a greater or lesser degree by the pre-treatment with clearing factor. Addition of a mixture of clearing factor and toxin to clear serum gave a response equal in magnitude to that given by toxin alone; so it may be presumed that there was no direct inhibition of the enzyme by clearing factor.

Of the second group, five gave a similar response to that outlined above, but in two cases there was a reversal of the effect, *i.e.* the increase in turbidity on incubation with the α-toxin was enhanced by prior treatment with clearing factor, such a result being given by one member of the first group also. It was suspected at first that this "abnormal" sensitization of these sera might be related to the presence of abnormal lipoproteins such as those which GOFMAN has associated with the atherosclerotic state. However, both the sera from confirmed atherosclerotics which we have examined so far have given "normal" responses, *i.e.* a suppression of the NAGLER effect. These results are summarized in Table III. It is of interest to note that the two abnormal responses of the clinical group were given by sera from patients with biliary cirrhosis, a condition which is known to be accompanied by a high concentration of an abnormal β-lipoprotein[14].

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TABLE III

THE EFFECT ON THE BREAKDOWN OF SERUM LIPOPROTEINS BY *Cl. welchii* α-TOXIN OF PRE-TREATMENT OF THE SERUM WITH "CLEARING FACTOR". ONE ml OF SERUM WAS INCUBATED WITH 2 ml OF A 1% (W/V) SOLUTION OF THE ACTIVE GLOBULIN FRACTION FOR 3 h AND 1 ml OF α-TOXIN ADDED. THE INCREASE IN TURBIDITY WAS FOLLOWED UNTIL THE PROGRESS CURVE FLATTENED

Subject	*Group*	*Serum phospholipids (mg/100 ml)*	*Serum total cholesterol (mg/100 ml)*	*Increase in turbidity*		
				Treated with CF	*Saline control*	
Normal response						% "inhibition" by clearing factor
O.T.G.	Normal	182.3	177.5	0.095	0.571	83.4
SB-2	Atherosclerotic	—	—	0.160	0.840	81.1
H.L.	Diabetic	400	402	0.145	0.605	76.0
E.G.	Cirrhotic	300	203	0.045	0.163	72.4
K.F.	Normal	206.5	190.4	0.100	0.355	71.8
T.H.	Sec. hep. carcin.	222	188	0.134	0.462	71.0
L.L.	Normal	114.6	187.5	0.248	0.789	68.7
W.G.B.C.	Normal	206.3	219.0	0.310	0.810	61.8
J.T.	Diabetic	140	195	0.090	0.210	57.2
A.B.	Diabetic	287	280	0.233	0.368	36.7
J.T.S.	Normal	292.3	277.4	1.30	1.84	29.4
R.T.B.L.	Normal	92.9	178.8	0.355	0.465	23.7
SB-7	Atherosclerotic	—	—	0.415	0.553	26.8
G.R.W.	Normal	177.2	182.1	0.796	0.972	18.1
M.H.	Normal	184.7	175.0	0.212	0.253	16.2
R.G.	Normal	212.8	212.8	0.305	0.359	15.0
Abnormal response						% "activation" by clearing factor
H.H.	Normal	187.5	171.7	1.25	0.605	107
W.H.	Biliary Cirrhosis	600	315	0.355	0.165	115
A.P.	Biliary Cirrhosis	*ca.* 1500	*ca.* 900	2.10	0.895	135

DISCUSSION

The kinetic studies so far carried out do not provide any further information on the mechanism of this transformation, although they may provide a basis for future investigations into factors which may influence the course of chylomicra dissolution.

The studies here reported on the interaction of the effects of clearing factor and of the phospholipase of *Cl. welchii* toxin provide an experimental basis for the integration of a number of hitherto isolated pieces of knowledge concerning the lipoprotein complexes of plasma. On the basis of the facts reported in the preceding section we may now write as an alternative to the scheme contained in the introduction, the following:

Chylomicra
↓ CF
Phospholipase Sensitive Lipoprotein
↓ CF
Phospholipase Insensitive Lipoprotein

However, an examination of the literature concerning the structure of the β-, and α-lipoproteins suggests that there may be no difficulty in equating the two summaries.

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PETERMANN[22] has demonstrated the liberation of lipid by *Cl. welchii* toxin from β-lipoprotein isolated by ultracentrifugation, and both MCFARLANE[23] and ONCLEY, GURD AND MELIN[24] have pointed out that it is necessary to presume the presence of phospholipid in the surface of the spherical β-lipoprotein molecule. This necessity is shown by a comparison of its chemical composition and its molecular dimensions. A simple calculation shows that there is not sufficient protein to form an intact all-protein surface and that there must therefore be lipid in this surface. As however the molecule is hydrophilic this nonprotein element of the surface must be polar in nature and is therefore presumably phospholipid.

An analogous calculation for the α-lipoprotein, which is an ellipsoid of revolution in form, does not appear to have been made and indeed such calculations must involve a number of uncertainties as pointed out by LUCK[25]. We have however performed this calculation. The volume of the lipid moiety is readily calculated and by assuming the protein surface film to be of uniform thickness one may set up simultaneous equations in which the unknowns are the major and minor semi-axes of the lipid portion. In this way the surface area of the lipid residue may be calculated and when this is done it is found to equal 17,200 A^2. However, when allowance is made for the thickness of the protein monolayer it is found that the area over which protein would have to be spread is 24,300 A^2. The area which could be covered by the protein moiety actually present if spread as a compressed film is 17,400 A^2 and it is therefore necessary to assume the presence of phospholipid in the surface of the α-lipoprotein molecule also. However, the area of phospholipid in the exposed surface of each molecule (6,900 A^2) is only a quarter of that present in the surface of the β-lipoprotein. This may be of prime importance in the explanation of our results.

COHN[26] has suggested that the sensitivity of a lipid-protein complex to *Cl. welchii* phospholipase is to be correlated with the presence of phospholipid in the surface of the complex. If we accept this apparently reasonable suggestion, the only assumption necessary to equate the findings here reported with the evidence obtained by ultracentrifugation is that the reactivity of any complex to *Cl. welchii* phospholipase will be governed by the actual area of phospholipid exposed on the molecular surface.

If such an assumption is made the above results may be regarded as providing confirmation of the findings reported in references 6, 7, and 13, the production of α-lipoprotein from β-lipoprotein under the influence of clearing factor being accompanied by a decrease in sensitivity to the phospholipase of the toxin. Conversely if the two schemes are assumed to be equivalent the inhibition of the NAGLER effect by clearing factor here reported may be regarded as the first direct evidence in confirmation of the structures assigned to the α- and β-lipoproteins on physico-chemical grounds. In either way a consistent picture of plasma lipoprotein interrelationship is reached.

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SUMMARY

The concentration of the lipaemia-clearing factor of heparinised plasma performed by ANFINSEN *et al.* has been repeated. The kinetics of lipaemia-clearing *in vitro* have been examined and the interrelationships of this clearing effect with the production of turbidity in human sera by *Clostridium welchii* α-toxin have been investigated. Pre-treatment of lipaemic sera with this toxin abolishes the clearing effect. Pre-treatment of human fasting sera with clearing factor suppresses the increase of turbidity brought about by subsequent incubation with the toxin. These results are discussed in the light of current conceptions of the structure of plasma lipoproteins.

RÉSUMÉ

La concentration du facteur clarifiant la lipémie dans le plasma héparinisé a été achévé par ANFINSEN *et coll.* et nous l'avons répété. La cinétique de la clarification *in vitro* a été examiné et les relations entre cet effet clarifiant et la turbidité causé par la toxine α du *Clostridium welchii* dans le serum humain ont été le sujet de nos investigations. Cet effet clarifiant est aboli par un traitement préalable du serum lipémique par la toxine. L'augmentation de la turbidité causé par incubation du serum humain jeûne avec la toxine est diminué par traitement préalable avec le facteur clarifiant. Ces résultats ont été mises en relation avec les conceptions courants sur la structure des lipoprotéines dans le plasma.

ZUSAMMENFASSUNG

Die von ANFINSEN *et al.* beschriebene Anreichung des Lipämie-Klär-Faktors aus heparinisiertem Plasma wurde wiederholt. Die Kinetik der Lipämie-Klärung wurde *in vitro* untersucht. Die Beziehung dieses Klär-Effekts zur Trübung menschlichen Serums durch *Clostridium welchii* α-Toxin wurde studiert. Vorbehandlung von Lipämie-Serum mit diesem Toxin bringt den Klär-Effekt zum Verschwinden. Vorbehandlung menschlicher Nüchtern-Sera mit dem Klär-Faktor unterdrückt die durch darauffolgende Inkubation mit dem Toxin verursachte Zunahme der Trübung. Diese Ergebnisse werden mit der bestehenden Auffassung über die Struktur der Plasma-Lipoproteine in Zusammenhang gebracht.

REFERENCES

1 P. F. HAHN, *Science*, 98 (1943) 19.
2 C. B. WELD, *Proc. Can. Physiol. Soc.*, (1948) 39.
3 N. G. ANDERSON AND B. FAWCETT, *Proc. Soc. Exptl. Biol. N.Y.*, 74 (1950) 768.
4 C. B. ANFINSEN, E. BOYLE AND R. K. BROWN, *Science*, 115 (1952) 583.
5 J. J. SPITZER, *Am. J. Physiol.*, 171 (1952) 492.
6 D. M. GRAHAM, T. P. LYON, J. W. GOFMAN, H. B. JONES, A. YANKLEY, J. SIMONTON AND S. WHITE, *Circulation*, 4 (1951) 666.
7 E. BOYLE, J. H. BRAGDON AND R. K. BROWN, *Proc. Soc. Exptl. Biol. N.Y.*, 81 (1952) 475.
8 R. L. SWANK AND V. WILMOT, *Am. J. Physiol.*, 167 (1951) 403.
9 R. L. SWANK AND S. W. LEVY, *Am. J. Physiol.*, 171 (1952) 208.
10 H. G. KUNKEL AND R. J. SLATER, *J. clin. Invest.*, 31 (1952) 677.
11 J. L. ONCLEY, G. SCATCHARD AND A. BROWN, *J. Phys. colloid Chem.*, 51 (1947) 156.
12 E. M. RUSS, H. A. EDER AND D. P. BARR, *Am. J. Med.*, 11 (1951) 468.
13 E. A. NIKKILA, *Scand. J. Clin. Lab. Invest.*, 4 (1952) 369.
14 E. J. COHN, L. E. STRONG, W. L. HUGHES, JR., D. J. MULFORD, J. N. ASHWORTH, M. MELIN AND H. L. TAYLOR, *J. Am. Chem. Soc.*, 68 (1946) 459.
15 J. L. ONCLEY, M. MELIN, D. A. RICHERT, J. W. CAMERON AND P. M. GROSS, JR., *J. Am. chem. Soc.*, 71 (1949) 541.
16 F. P. O. NAGLER, *Brit. J. Exptl. Path.*, 20 (1939) 473.
17 R. G. MACFARLANE, C. L. OAKLEY AND C. G. ANDERSON, *J. Path. Bact.*, 52 (1941) 99.
18 M. G. MACFARLANE AND B. C. J. G. KNIGHT, *Biochem. J.*, 35 (1941) 884.
19 A. C. FRAZER, J. J. ELKES, H. G. SAMMONS, A. D. T. GOVAN AND W. T. COOKE, *Lancet*, 1 (1945) 457.
20 E. H. AHRENS, JR. AND H. G. KUNKEL, *J. Exptl. Med.*, 90 (1949) 409.
21 A. BOGOCH, W. G. B. CASSELMAN, A. KAPLAN AND H. L. BOCKUS, *Gastroenterology*, 23 (1953) 516.
22 M. L. PETERMANN, *J. Biol. Chem.*, 162 (1946) 37.
23 A. S. MCFARLANE, *Disc. Faraday Soc.*, 6 (1949) 74.
24 J. L. ONCLEY, F. R. N. GURD AND M. MELIN, *J. Amer. chem. Soc.*, 72 (1950) 458.
25 J. M. LUCK, *Disc. Faraday Soc.*, 6 (1949) 93.
26 E. J. COHN, *Disc. Faraday Soc.*, 6 (1949) 93.

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