diverse sources and of a wide range of activity, is so high, is very strong evidence that all three tests are, in fact, measuring the activity of the same agent. In view of the contradictory reports that have appeared in the literature it is interesting to note, that when the viscosimetric assay is carried out at pH 7.0, in approximately physiological salt concentration, very good agreement is obtained with the results of skin-diffusing activity.

SUMMARY

1. A method of assaying hyaluronidase is described, which depends upon the destruction by the enzyme of the power of a substrate-protein complex to form a typical 'mucin clot' on the addition of acetic acid.

2. It appears that the destruction of the clotting power of the substrate is an early stage in its degradation, which can be detected before any appreciable fall in viscosity occurs.

3. Sera which inhibit the diffusing and viscosity-reducing activity of these enzymes also inhibit the mucin-clot prevention reaction. Sera prepared against enzymes obtained from *Cl. welchii* and *Vibrio septique* are species- but not type-specific. Those obtained against streptococcal enzymes are group- but not type-specific. A serum prepared against diffusing factor from bull's testis inhibited this enzyme, but did not inhibit a testicular enzyme from the mouse, or any of the bacterial enzymes.

4. Both the apparent pH optimum of viscosity-reducing activity, and the titre of the enzyme, are markedly affected by the concentration and nature of salts in the system.

5. Nine enzymes from various sources have been examined for diffusing activity in the skin, by viscosimetry and by the mucin-clot prevention test. The conclusion is drawn that all three tests measure the activity of the same agent.

I wish to express my appreciation of the keen cooperation and many constructive suggestions of Mr C. W. Hale.

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Studies on Diffusing Factors

3. A NEW BIOLOGICAL ASSAY OF DIFFUSING FACTOR IN GUINEA-PIGS

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The most accurate biological method of assaying diffusing factor hitherto published is that of Bacharach, Chance & Middleton [1940]. This method compares the activities of pairs of preparations by measurement of the mean bleb size produced in suitable rabbits, using a 'four-point' assay. Since the log dose-response curve is sigmoid these authors found that the four doses must be carefully adjusted so as to lie upon the straight portion of the curve, but if such precautions are observed they claim to be able to distinguish satisfactorily between doses

in the ratio of 2 to 1. In the course of work undertaken to establish the identity, or otherwise, of diffusing factor and hyaluronidase, it became desirable to be able to detect twofold differences in diffusing-factor activity. In view, however, of the difficulty caused by the non-linearity of the log dose-response curve [Evans & Madinaveitia, 1940], it was decided, at the suggestion of Mr C. W. Hale, to devise an accurate assay of the least dose which would produce a significant increase in bleb area in a given time, when injected intracutaneously

into a group of guinea-pigs. The principle is the same as that underlying tests of minimal lethal dose, etc. The time chosen, which is the shortest convenient, is also such that active diffusion is still taking place when the bleb size is measured, and the measurement is made before the initial velocity of reaction has fallen significantly. The method to be described has provided, in our experience, an absolute assay, inasmuch as the minimal diffusing dose is the same for repeated assays of the same preparation, using different batches of animals. With reasonable precautions in the choice of animals, and within the limits of accuracy of the test, it should be possible for different workers to use comparable units without necessarily sharing a common standard preparation.

METHODS

Procedure for assay

General. Two control and 6 test doses of diffusing factor (D.F.) are injected intracutaneously into each of 6 guinea-pigs, with haemoglobin as an indicator of the bleb size [Madinaveitia, 1938]. After exactly 20 min. each animal is killed and the skin of the back is rapidly flayed. The areas of the blebs are measured on the underside of the skin, and the minimal diffusing dose is taken as the least amount of D.F. which will produce a 20% increase in mean area as compared with the mean of the controls.

Animals. The guinea-pigs should weigh over 300 g. and their skins should be white. For each assay 7 are required, and their backs are clipped before use.

Solutions. The dilutions of D.F. are made in physiological saline shortly before injection. To each dilution is added an equal volume of horse haemoglobin solution, made by washing horse red cells with physiological saline six or seven times on the centrifuge, laking by the addition of distilled water up to the original volume of the blood, and making up to 0.85% (w/v) NaCl by the addition of solid NaCl, in quantity calculated from the chloride content of the solution. The final dilution of D.F. is thus 1:2 of the original. It is important to wash the cells very well, since horse serum inhibits the action of D.F. and it is best to add the haemoglobin solution after making the dilutions, since it is viscous and renders accurate pipetting difficult.

Injections. 0.2 ml. of each dilution, and two controls of 0.2 ml. of equal parts haemoglobin solution and saline, are injected intracutaneously with Nimrod syringes into each guinea-pig. The position on the guinea-pig does not matter (see below), but the order should be kept constant to facilitate reading later. A separate syringe should be used for each dilution. It is convenient to inject one guinea-pig every 3 min., since the first is then ready for killing and measurement soon after the last one has been injected. There is a small leak back from each bleb, but under standard conditions this is fairly constant in amount.

Measurement of blebs. Exactly 20 min. after injection each animal is killed by a blow on the head, and the skin containing the blebs is removed from its back. The skin is then laid out flat with the inside uppermost and sponged with cold water to clean the skin and cause the muscular layer to relax. The blebs stand out sharply, and are elliptical, except for those in which very considerable diffusion

has occurred. The area is obtained by measuring the shortest and the longest diameters with dividers and multiplying their product by $\frac{\pi}{4}$. Since the time taken for measurements on each guinea-pig is 3 min. or less the time-table can be adhered to fairly accurately.

A preliminary orientating assay is made in a single guinea-pig using six tenfold dilutions of D.F. Having found roughly what dose gives a 20% increase in bleb area over the controls, the accurate assay is carried out using six twofold dilutions of D.F. adjusted to cover the end-point arrived at in the preliminary assay. Examples are given in Table 3.

RESULTS

The test described above was worked out as the result of an experiment in which each of 6 guinea-pigs was given 8 intracutaneous doses of 0.2 ml. haemoglobin-saline in a constant order, and bleb area measured in mm.² after 20 min. (Table 1).

Table 1. *Variation in area of blebs in control experiments without diffusing factor*

	Animal no.					
	I	II	III	IV	V	VI
	Size of individual blebs (mm. ²)					
	159	165	195	163	170	206
	180	164	187	200	176	214
	180	143	181	204	201	212
	170	162	188	159	177	214
	176	177	195	189	—	187
	177	174	201	176	181	176
	153	194	176	179	189	201
	167	194	174	193	195	—
Means	170	172	187	183	184	201
Greatest deviation from mean as % of mean: Plus	6	13	7.5	11.5	9	6.5
Minus	10	17	7	13	7.5	12.5
Coefficient of variation	5.5	9.3	4.5	8.4	4.6	6.8

From the results in Table 1 it can be seen that although most of the guinea-pigs have a similar mean bleb size, occasionally one differs from the others. The size of individual blebs on any one guinea-pig was fairly constant, and appeared to grow more constant as our skill improved in later tests. There is no constant variation with position on the animal. If a variation equal to twice the standard deviation is considered significant, it appears that in each animal a 20% increase in bleb size, compared with the mean for that animal, would be significant. In our test by taking 2 blebs on each of 6 guinea-pigs as a control we have 12 control blebs against which to compare 6 test blebs for each dose level.

A similar experiment to the above was done with a known diffusing dose, to ascertain whether the same degree of constancy could be obtained when diffusion was occurring. One minimal diffusing dose of preparation G3 was used throughout. This, in

previous assays at this dose level, had given mean percentage increases in area of 20 and 25 %. In this test the size of the control blebs showed fair constancy and there was fairly good agreement between test doses on any one guinea-pig—apart from one bleb in each of animals IV and V, which differ markedly from the others, and which increase considerably the coefficient of variation. There was definite varia-

paration, which can be detected by our test at a dilution of 1 : 40,000. It will be seen that in the critical range the agreement of the mean values is quite good, despite considerable variation in individuals.

In the first 10 tests which we made, comprising a total of 60 guinea-pigs, an analysis was made of the individual responses as compared with the

Table 2. Variation in area of blebs with one minimal diffusing dose of diffusing factor

	Animal no.					Combined totals
	I	II	III	IV	V	
	Bleb areas (mm. ²)					
	240	214	320	269	276	Mean test bleb 257
	254	213	290	276	254	
	262	227	252	(330)	268	
	254	213	267	254	240	
	248	212	310	254	(203)	
	230	228	266	274	314	
Control blebs	198	188	200	201	200	Mean control bleb 201
	212	200	214	200	200	
Mean percentage increase over controls	21	12	37	38	29	28
Coefficient of variation for test blebs	4.3%	3%	8.9%	9.8%	13.6%	For all test blebs 12.7%

Table 3. Data for two separate assays on the same preparation of diffusing factor

(a) Preliminary assay of diffusing factor on single guinea-pig

Dilution of D.F.	Bleb area mm. ²	Increase over controls %	Dilution of D.F.	Bleb area mm. ²	Increase over controls %
1 : 10 ³	475	166	1 : 10 ⁶	189	6
1 : 10 ³	314	76	1 : 10 ⁷	201	13
1 : 10 ⁴	219	23*	Control	175	179
1 : 10 ⁵	206	16	Control	182	

(b) Complete test on groups of guinea-pigs

Animal no. ... Dilution of D.F.	First test						Second test					
	1	2	3	4	5	6	7	8	9	10	11	12
	Bleb areas (mm. ²)											
1 : 5,000	227	297	314	329	304	348	277	269	294	245	410	307
1 : 10,000	249	262	260	262	227*	284	255	283*	232	252	314	226
1 : 20,000	254*	255	262	255	189	—	213	176	255	214	283*	280
1 : 40,000	212	259*	258*	269*	187	299	245*	176	218*	214*	190	208*
1 : 80,000	182	171	176	170	213	299*	189	187	176	165	185	177
1 : 160,000	224	188	201	201	205	227	165	201	149	182	171	177
Control	192	232	173	163	194	205	176	165	182	153	180	189
Control	176	212	200	154	—	214	160	156	143	171	189	154

* Greatest dilution showing 20% increase in area over controls.

(c) Summary: Mean for six guinea-pigs

	First test	Second test		First test	Second test
Controls	192	167	1 : 20,000	233 (plus 21%)	237 (plus 42%)
1 : 160,000	208 (plus 8%)	174 (plus 4%)	1 : 10,000	258 (plus 35%)	260 (plus 56%)
1 : 80,000	202 (plus 5%)	180 (plus 8%)	1 : 5,000	305 (plus 59%)	300 (plus 80%)
1 : 40,000	230 (plus 20%)	209 (plus 25%)			

tion in sensitivity from animal to animal, but the average amount of diffusion for all pigs at this dose level (28 %) agrees well with previous assays of the same preparation (Table 2).
In Table 3 is given an example of two separate complete assays of a testicular diffusing-factor pre-

averaged response of each group of 6 animals. The results (Table 4) showed that 50 out of 60 animals gave an end-point within one twofold dilution on either side of that dilution which was the end-point deduced from the mean response of the batch.

Table 4. Scatter of assay results with 60 animals

Assigned dilution	No. of animals giving end-point at					
	1 dilution		2 dilutions		3 dilutions	
	Less	More	Less	More	Less	More
31	11	8	6	2	1	1

With the kind co-operation of Mr Bacharach and his co-workers a series of preparations was assayed by our method, by the method of Bacharach *et al.* [1940], and by a modification of the viscosity-reduction test described by McClean & Hale [1941] which we believe to be accurate to within 10%. The results are listed in Table 5, in which we have

Table 5. Comparative results with three methods of assay

Enzyme preparation	Diffusing activity in Glaxo units/ml.*		Viscosity reducing activity units/ml.
	Our assay	Glaxo assay	
<i>Streptococcus</i> C7 (purified)	750	4,650	12
Glaxo standard testis (1 mg./ml.)	2,000	(2,000)	29
<i>Cl. welchii</i> (purified)	4,000	4,050	57
<i>Cl. welchii</i> (filtrate)	8,000	4,800	57
Testis (P.M. 1)	8,000	12,400	125
<i>Streptococcus</i> (A4 filtrate)	10,000	19,600	93
<i>Streptococcus</i> (C7 filtrate)	10,000	13,500	225
Testis (G. 3)	200,000	19,000	1,280
<i>Cl. welchii</i> (W. 74 purified)	800,000	60,300	15,150

* The conversion of our assay into Glaxo units is carried out as follows: for the Glaxo Standard testis powder, by their definition, one diffusion unit (D.U.) is contained in 0.5 μ g. We found on repeated test that a solution of this powder containing 1 mg./ml. showed significant diffusion at a highest dilution of 1:400 of the solution. Our minimal diffusing dose (M.D.D.) therefore contains 0.25 μ g. and hence 2 M.D.D. corresponds to 1 D.U.

calculated our minimal diffusing doses so as to give the potency of the preparations in terms of the Glaxo Standard, which we assayed by our method. A full discussion of these and other results is given elsewhere [McClean, 1943], from which it will be seen that, within the limits of the tests, there is fair

agreement between our minimal diffusing dose assay and the viscosity-reducing activity, whereas with the Glaxo assays the agreement is less good. It is only necessary here to discuss the degree of agreement between the two biological methods of assay. Over the range 2000–10,000 units/ml. this is good, but with the weaker and the stronger preparations the differences are far outside the experimental errors of the two tests.

DISCUSSION

The method of Bacharach *et al.* [1940] is based upon the measurement, in the skins of rabbits, of the total area through which a preparation spreads in a given time, compared with the area of spread of a standard preparation. The areas of diffusion are proportional to the logarithms of the doses, only over a limited range of dilutions, which these workers select for their test. Their method depends upon the evaluation of the slope of the log dose-response curve (*b*) at that part of the curve at which it is linear, and they state that the values of *b*, for the standard and for the test blebs, are, in any one experiment, always sufficiently close to permit of accurate calculation. The method described above measures the smallest dose of D.F. which produces significant increase in diffusion, and measures therefore a quantal response. It has in its favour that it is relatively easy to carry out in practice. Statistically, however, it is less satisfactory than the analysis of a continuous variate, as carried out by Bacharach *et al.* [1940]. Yet the results of our assay are in closer agreement than theirs with the determinations of viscosity-reducing activity, when the latter is measured under physiological conditions of salt concentration and pH [McClean, 1943]. Re-examination of all the assays, with a 50% increase in bleb area over the mean controls instead of a 20% increase as the critical response, yielded a similar picture (Table 6). Unlike the method of the Glaxo workers, no standard preparation is included in each test, and therefore analysis requires the construction of a standard log dose-response curve

Table 6. Comparison of results of assays based on measurements of 20% and of 50% increases in area of bleb

Enzyme preparation	Viscosity- reducing units/ml.	D.U.* (20% increase)/ml.		D.U.* (50% increase)/ml.	
		D.U./V.R.U.*		D.U./V.R.U.*	
<i>Streptococcus</i> C7 (purified)	12	750	63	500	42
Standard testis	29	2,000	69	2,000	69
<i>Cl. welchii</i> (eluate)	57	4,000	70	2,000	35
<i>Cl. welchii</i> (crude)	57	8,000	140	2,000	35
Testis P.M.	125	8,000	64	4,000	33
<i>Streptococcus</i> A4	93	10,000	107	5,000	53
<i>Streptococcus</i> C7 (crude)	225	10,000	44	10,000	44
Testis G3	1,280	200,000	156	100,000	77
		Two assays		50,000	38
<i>Cl. welchii</i> 74	15,150	800,000	52	200,000	13

* D.U. Glaxo-diffusing unit. V.R.U. viscosity-reducing unit.

for guinea-pigs under the conditions described. This was attempted, using the figures obtained in all the various assays performed, but the resulting values for the slope of the best straight line (*b*) were so divergent as to render further calculation useless. It is perhaps surprising, in these circumstances, that the method should give results which are apparently more consistent with other properties of the preparations used. We have been unable to arrive at any satisfactory explanation of the difference between the tests, and can only offer a tentative suggestion that, for the biological assay of D.F., the guinea-pig is a more satisfactory animal than the rabbit.

SUMMARY

A method is described for assaying the activity of diffusing-factor preparations in the skin of guinea-pigs, whereby twofold differences in activity can be distinguished. This method, which is based on the determination of the least amount required to produce a 20% increase in bleb area, gives reasonable agreement with assays of the hyaluronidase content of the same preparations, when this is estimated by the reduction of the viscosity of hyaluronic acid.

I wish to acknowledge the assistance of Mr C. W. Hale who suggested the investigation of this method of test and collaborated throughout.

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A Method for the Estimation of Glucose-1-phosphate and Galactose-1-phosphate in the Liver

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The estimation of glucose-1-phosphate and galactose-1-phosphate in solutions containing only small quantities of inorganic phosphate and reducing substances does not offer any particular difficulties. Both esters are readily hydrolysed by acid and the liberated inorganic P and hexose can easily be determined [Cori & Cori, 1937; Kosterlitz, 1938]. In the liver, however, relatively large quantities of inorganic P (about 30 mg. P/100 g. liver) and considerable quantities of reducing substances (corresponding to 300 mg. glucose/100 g. liver) make it difficult to determine such small quantities of hexose-1-phosphates as may be expected to be present. The acid-soluble organic P fraction obtained from rabbits' livers during galactose assimilation contained only 5–12 mg. galactose or 0.9–2 mg. P/100 g. liver as galactose-1-phosphate and less than 1 mg. glucose or 0.17 mg. P/100 g. liver as glucose-1-phosphate [Kosterlitz, 1939b]. Furthermore, the glycogen present in the liver yields glucose on acid hydrolysis and also interferes with the fractionation of the soluble and insoluble Ba salts. For these reasons, a preliminary step in the estimation of the 1-esters in trichloroacetic acid extracts of liver is

the removal of the interfering substances, viz. inorganic P, reducing substances, and glycogen.

A description of the method as it has been used for the determination of the hexose-1-phosphates present in the livers of rats fed with glucose or galactose will be followed by a critical examination of the various steps involved in the determination.

METHODS

The excised liver is rapidly washed free from adherent blood, dried between filter paper, and cut into 4–5 pieces which are immediately immersed in liquid oxygen. When frozen, 5 g. or more are weighed and transferred to an ice-cold mortar containing 20 ml. 5% ice-cold CCl_3COOH and some acid-washed sand. Throughout the procedure, whenever the reaction is acid, all glass vessels and reagents are kept as near 0° as possible. After the liver has been thoroughly ground, additional 5% CCl_3COOH is added in quantity such that each 10 ml. of the final extract represent 1 g. of wet liver. In these experiments the water content of the liver has been assumed to be 80% of the liver weight. Therefore, a total of 9.2 ml. 5% CCl_3COOH /1 g.