47. A Biological Test for Vitamin P Activity

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The absence of a satisfactory animal test for vitamin P has had several disadvantages. Clinical trials have been made not only with hesperidin but also with citrus concentrates that must have contained at least one other active substance and may even have been devoid of hesperidin. This compound, although its chemical structure is established, cannot itself be estimated by any sufficiently specific chemical or physical tests. Thus the vitamin P activity of the concentrates used, and possibly also of the hesperidin, can only be expressed in terms of the clinical results attained; such expression is of very limited precision, and is, moreover, dangerously liable to lead to argument in a circle. Furthermore, attempts to concentrate the active principle from natural sources, to say nothing of isolation and synthesis, have been handicapped or frustrated by the inability to relate chemical properties with physiological activity. The same difficulties have naturally also impeded clinical investigation, and the pioneer work of Armentanp, Bentsáth, Beres, Rusznyák & Szent-Györgyi [1936] and of Scarborough [1939] has been followed by only a few other trials [cf. Rapaport & Klein, 1941; Goldfarb, 1941]. Even their limited results, however, have given a more than academic interest to the problem of producing experimental avitaminosis-P in laboratory animals.

The early attempts of Szent-Györgyi and his co-workers [Bentsáth, Rusznyák & Szent-Györgyi, 1936; 1937] to demonstrate vitamin P deficiency in guinea-pigs were not found convincing: Zilva [1937] and Moll [1937] were both unable to confirm Szent-Györgyi's results, and Szent-Györgyi [1938] was himself unable to repeat them later. We were therefore interested in a paper by Zacho [1939] who used as a criterion of vitamin P deficiency the 'specific' symptom of capillary fragility, sometimes confused, as Scarborough [1941*a*] has pointed out, with increased capillary permeability. The criteria used by the earlier investigators—increased survival time or a reduced number of haemorrhages in scorbutic guinea-pigs—were clearly unsatisfactory even on theoretical grounds: death of guinea-pigs from scurvy is complicated by intercurrent infections, and estimation of the animals' haemorrhagic 'score' *post mortem* must be largely a matter of subjective judgement. Both would be accompanied by a high variance, so that clear-cut results would be difficult to get even with large numbers of animals.

Interest in Zacho's paper has also been aroused in the Hungarian workers, and Rusznyák & Benkó [1941] have stated that they were able to confirm Zacho's findings, not only on guinea-pigs but also on rats. When their note appeared, we had already overcome certain difficulties encountered in trying to apply Zacho's technique, but we had considered it inadvisable at that stage to publish our results because we wished to try to put the method on to a quantitative basis, in our opinion the acid test of any claim for an animal response to a physiologically active substance.

Without intending to detract at all from Zacho's findings, which we have been able to confirm in principle, if not in every detail, we wish briefly to record our criticism of his method and results. First, his figures were published in such a way as not to allow any estimate of the errors involved in his procedure. Secondly, he used animals of extremely varied weights (240–685 g.) and therefore, presumably, of correspondingly varied ages. Thirdly, he applied a depilatory and took readings of capillary fragility every second or third day; both these procedures, in our experience, seriously increase the difficulty of

'reading' the petechiae produced by the negative pressure method. Moreover, too frequent examination of the animals is undesirable; after each shave the new growth of hair becomes progressively coarser, making it progressively more difficult at the next shave to remove the hair without so damaging the skin as to interfere with accurate detection of petechiae. Finally, he administered only 'citrin', prepared by the method of Szent-Györgyi, but from rose-hips, and gave all doses parenterally, a proceeding impossible with hesperidin and therefore unsuited for making comparisons between the activities of his concentrate and the pure glycoside.

Our procedure differs from Zacho's in a number of details, as will be apparent from the following description of the methods used in the work described here. Healthy young albino guinea pigs, weighing from 180 to 320 g., were placed on a scorbutogenic diet of bran, crushed oats and separated milk powder. All animals received 1 ml. of cod-liver oil twice weekly and 5 mg. of pure ascorbic acid every day except Sundays orally by pipette, direct into the animal's mouth; the material to be tested was similarly administered, either in a well-mixed aqueous suspension (e.g. hesperidin) or in aqueous solution. The latter contained a solid complex of citrus glycosides with calcium, prepared for us by the Research Department of the California Fruit Growers Exchange and placed at our disposal by the courtesy of Mr C. P. Wilson and Mr W. E. Baier, to whom we are much indebted. We learned from them that this concentrate, which was a yellow powder, hardly at all hygroscopic and almost completely soluble in water to give a brownish-yellow solution, was made from orange meal, i.e. the peel and pulp of oranges freed from juice and dried in warm air, by the method described by Szent-Györgyi for preparing 'citrin', though with some modifications.

The chemical nature of these glycosidic fractions is quite unknown; the belief that they contain 'eriodictin' [Szent-Györgyi, 1938], that is, demethylated hesperidin, can only be justified by acceptance of Szent-Györgyi's statement that this is the water-soluble constituent of citrin: for this statement he has never published any evidence. We propose, therefore, to refer to these mixtures as water-soluble vitamin P preparations, more briefly as w.s.p. 1, w.s.p. 2 and so on.

The animals are prepared for test by clipping the hair on the median part of the back and then shaving the skin over an area about 2 cm. wide on either side of the lumbar vertebrae. The skin is greased with vaseline and the animal placed in a warm room (at a temperature of $70 \pm 2^\circ$ F.) for 1-2 hr. before removal and examination for capillary fragility. This 'preheating' ensures a good blood supply to the skin and easy observation of any petechiae produced by suction and gives more consistent responses. Animals held at a low temperature have only shown very faint petechiae even with a negative pressure of 200 mm. The guinea-pig appears to respond to cold with increased capillary resistance.

In passing, two more or less casual observations may be recorded; they appear to confirm the fact, to which Scarborough [1941 b] has called attention, that the presence of extra-vascular blood may lead to increased capillary resistance. One batch of animals responded very poorly to the diet, that is to say, their capillary resistance remained much higher than that of previous and subsequent batches similarly treated. They were in poor health; a number died before and others during the experimental period. Bacteriological examination revealed the presence of *pseudo-tuberculosis rodentii*, suggesting that lung haemorrhages may very likely have been present. A few other animals were rather badly cut during shaving; they also failed to respond and maintained abnormally high capillary resistances. Similarly, the animals of Zilva, Moll and Szent-Györgyi may have been protected from increased capillary fragility as a response to their own scorbutic ecchymoses.

The apparatus used for applying 'negative pressure' to the shaven skin of the animal is shown diagrammatically in Fig. 1; it is a simplified version of the instrument used by

Scarborough for measuring capillary resistance in human subjects. The open end of the suction cup has a diameter of 12 mm. (Zacho used one of 8 mm.), and the upper face is covered with optically plane glass. Replacement of the three-way tap used in our first apparatus by the needle valve has since made it possible to read pressures to the nearest 5 mm. instead of, as formerly, the nearest 10.

The suction cup is applied to the greased and shaven area and the pressure is gradually reduced by 5 mm. stages, being maintained at each stage for 3–5 sec. That pressure is noted at which petechiae are first seen when the area under suction is being viewed through the cup. This pressure has sometimes been called the 'capillary resistance', because the higher the pressure the higher the resistance; we consider the description, however, to be somewhat illogical and prefer to speak of the 'critical petechial pressure' (C.P.P.). Two observers take readings of each C.P.P., and the mean of their readings is used in all evaluations. A study of the contributions made to the variance of readings by such factors as position on the animal's back, uncontrollable animal differences and differences between observers is being undertaken. Whether or not such study leads to improvements in technique the readings already have an unusually small variance, as will be seen below.

The procedure described above was standardized after a large number of preliminary observations, the details of which would, however, be wearisome to follow here. The justification for the procedure must, in any event, be found in the degree of precision it allows.

In Table 1 are recorded the results obtained with three different doses of recrystallized hesperidin, compared with a control group untreated with vitamin P, and the corre-

Fig. 1.

Table 1

Substance	Dose mg.	Critical petechial pressure (mean of 10 or 9 animals), mm. Hg, after weeks				
		0	1	2	3	4
Hesperidin (recryst.)	0	97.5	78.0	63.0	58.0	60.0*
	5	92.0	73.0	67.2†	67.8	66.1
	10	97.0	83.0	72.5	71.7†	72.2
	20	87.0	78.0	72.5	75.5	76.5
W.S.P. 1	0	96.5	84.0	67.0	85.0	69.0†
	1	96.5	86.7*	72.8	90.6	78.9
	2	91.0	84.5	82.5	93.3†	87.8
	4	92.0	96.0	89.4	96.1	95.0

Scorbutogenic diet + 5 mg. ascorbic acid per animal per day.

* Returned to 93.9 after 21 days with 20 mg. hesperidin.

† One animal died.

‡ Returned to 95.5 after 14 days with 4 mg. W.C.P. 1.

sponding results obtained with the water-soluble preparation mentioned above. (In this experiment the apparatus was still fitted with the three-way tap and individual readings were only taken to the nearest 10 mm.) It will be seen that response is graded to dose at

each week, though the differential effects of the doses (an expression of the slope of the dosage-response curve) fluctuates markedly. This is particularly noticeable after 3 weeks' dosing with w.s.p.1; nevertheless, in spite of the sudden unexplained rise in C.P.P. at this point, there is no crossing of the curves relating the C.P.P. values with the different doses and these values fall again to the anticipated levels at the end of the 4th week.

Attention is also called to the steady and fairly rapid rise in C.P.P. shown by the negative control animals when vitamin P is administered.

It is evident from the figures shown in Table 1 that the maximum differences between the effects of different doses were obtained after 4 weeks; an analysis was therefore made, by the usual procedure, to establish the significance of these differences; the results are shown in Table 2.

Table 2. *Effect of vitamin P on capillary resistance*

Substance	Daily dose mg. (d)	No. of animals surviving out of 10	Response (C.P.P.), mm. Hg				Significance of differences	
			Mean = $\bar{y}$	Variance = Σ^2	Coefficient of variation = $100\Sigma/\bar{y}$ %	Standard error of mean = $\sigma_{\bar{y}}$		
Hesperidin recryst. (93 %)	0	10	60.0	11.11	5.6	1.05	2.2	0.05
	5	9	66.1	61.13	11.8	2.61	2.0	0.07
	10	9	72.2	25.75	7.0	1.69	1.9	0.08
	20	10	76.5	22.50	6.2	1.50		
w.s.p. 1	0	10	69.0	54.44	10.7	2.33	3.2	<0.01
	.1	9	78.9	36.13	7.6	2.00	3.4	<0.01
	2	9	87.8	25.75	5.8	1.69	2.7	0.013
	4	9	95.0	37.50	6.4	2.04		

Period of test: 14 days. All animals received 5 mg. ascorbic acid on 6 days each week.

Regression line equations:

For hesperidin $y = 54.46 + 17.15 \log d$, $b/\sigma_b = 3.77$ ($P > 0.001$);

For w.s.p. 1 $y = 79.15 + 26.76 \log d$, $b/\sigma_b = 5.93$ ($P > 0.001$).

A number of conclusions may be drawn as to the suitability of the method for purposes of biological assay. They relate to the variability of the response and the slope of the dosage-response curve, as well as to the range of responses over which the method seems likely to be applicable.

First, it should be noted that the variance of response to dose is small and apparently independent of the level of response within the range used: both these are desirable characteristics in biological assay. The surprising smallness of the variance is indicated by the coefficients of variation, which are always independent of the unit of measure adopted. The values range from 5.6 to 11.8, and are thus exceptionally low; most biological methods give values over 20 %, often over 30 %.

Secondly, the slope of the response curves would appear to be satisfactory at the higher levels of response found with w.s.p.1. With doses in the ratio of 2:1, all pairs of mean responses show highly significant differences. At the lower levels of response given to hesperidin, significance is rather less than is now required by bio-assayists, who prefer not to recognize those corresponding to a probability of less than 20 to 1.

Fig. 2.

If the results are plotted in the usual way, with the responses as ordinates and the logarithms of doses as abscissae (Fig. 2), these findings are illustrated graphically by (1) the closeness with which the two sets of points fall on their respective regression-lines, calculated by the method of least squares, and (2) the steeper slope of the line referring to the higher range of responses.

It seems likely, therefore, that under our conditions of test vitamin P activity may be estimated with a considerable degree of accuracy. It will be necessary, apparently, to conduct assays in which the test substance is compared in potency with a provisionally adopted standard preparation, on groups of about 10 animals, to use doses in the ratio of 2 : 1 and to choose these doses so that the mean C.P.P. values fall between about 70 and 95 mm. Hg. Further experience of the test may lead to an increase in precision, making it possible to obtain the same degree of accuracy with a smaller number of animals or to use a lower dose ratio.

It may be of interest to direct attention to one incidental problem that is possibly of wider bearing on biological assays in general. In the tests described each animal can act as its own control; values of the C.P.P. are taken at the beginning and at the end of the test period. The 'metameter'* of response used in the analyses reported above was the reading of the C.P.P. at the end of the test period (y). If this were subtracted from the reading at the beginning of the period (z) the metameter would be ($z - y$), and it seemed to us obvious that this should be a better value to use, because it would tend to diminish the contribution made by animal variation to the total variance of response. This view was strengthened by our finding that y and z showed a high correlation in certain dosage groups chosen at random. It can, moreover, be demonstrated that this correlation also involves the correlation of ($z - y$) with both y and z , when y and z are both lineally related to the logarithm of the dose used.

In certain 'four-point assays', the results of which will be reported elsewhere, we tested the above theoretical conclusion by calculating potency ratio (M) and its error (σ_M^2) alternatively for each metameter. To our surprise we found that the error was considerably greater when M was based on ($z - y$) than when it was based on the observed values of y . For this statistical paradox we can offer no explanation, but it is clearly at present justifiable to base assays on the simple metameter y .

A more detailed study of the test will involve investigating further the standard deviation of single observations, preferably by a suitably planned experiment and a subsequent variance analysis. More fundamental, however, is the problem that arises with several types of biological assay, and more especially with vitamin assays. The tests described in this paper, and certain comparative assays to be reported elsewhere, are based on a prophylactic procedure: experience of other vitamin assays shows that a curative technique in most instances provides more information for a given amount of labour and material, including animals. The adaptation of the method described to animals 'run out' of vitamin P should not involve serious difficulties and might well improve the test for routine purposes.

We wish to express our thanks to Dr Harold Scarborough, with whom we have had many valuable discussions of the vitamin P problem in general and these tests in particular, and to Miss H. Weil for technical assistance in the care and handling of the experimental animals.

* We are indebted to Prof. Lancelot Högben for suggesting this useful term to designate the measurement, or transformation of the measurement, used in evaluating biological tests.

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