days later, the animals were sensitized intradermally with neoarsphenamine (0.35 mg/kg) as described earlier. From the 28th day onward, these animals were tested intradermally, first at weekly intervals and later at intervals of 1 and 2 months over a total period of 21 weeks. In none of these animals could either a flare-up, a tuberculin-type reaction, or a generalized dermatitis be provoked; they showed only a primary toxic reaction.

A further group of eight animals was treated with neoarsphenamine intravenously before any intradermal injection. Twenty-one days later they were sensitized intradermally and from the 28th day onward they were tested weekly for 9 weeks. In these animals as well, only small papules (primary toxic reactions) were provoked, as seen in unsensitized controls, which contrasted with the large papules (tuberculin-type reactions) seen in sensitized animals already described.

Induction of tolerance in previously sensitized animals was studied in a third experiment in which a group of 28 animals was sensitized intradermally with neoarsphenamine as described. Fourteen showed a spontaneous flare-up 7 to 14 days later, and 23 became sensitized, as demonstrated by an intradermal test on the 21st day. On the 28th day, the 23 sensitized animals were given neoarsphenamine (60 mg/kg) intravenously, and 6 hours later they were given an intradermal dose (0.35 mg/kg). This test, as well as all subsequent tests performed, first at intervals of 1 week and later after 1 and 2 months over a period of 28 weeks, was completely negative. This state of immunological unresponsiveness is specific, since the intravenous injection of neoarsphenamine did not interfere with the immunological response to dinitrochlorobenzene in five animals sensitized concomitantly with both compounds.

In order to determine whether this state of tolerance is due either to an overloading with, or a long persistence of, the haptene in the body, or both, further experiments were performed with a varying interval between the intravenous and the intradermal injections. When 6 or 12 hours elapsed, all 31 animals used became permanently tolerant. When the interval was extended to 1 or 3 days, 7 of 12 animals became unresponsive. No tolerance was achieved in 17 animals when 7 or 14 days elapsed between both injections (confirmation of results obtained by Sulzberger).

It seems, therefore, that for this particular compound tolerance in previously sensitized animals is obtained only when an intravenous injection of the haptene is followed some hours later by an intradermal one. That tolerance may not be due to the persistence of haptene in the body is indicated by the fact that animals receiving injections at intervals of 6 or 12 hours are still tolerant when tested 14 days later, whereas those injected at intervals of 7 and 14 days showed sensitivity, the time available for elimination of the intravenously given haptene being the same in all groups. To determine if tolerance persists because of renewed haptene supply by repeated testing, the first control test was performed in further groups of similarly tolerant animals as late as 3 months after induction of tolerance. Again, all 23 animals used remained unresponsive.

In comparable experiments (*9*) which we performed in animals sensitized to dinitrochlorobenzene (contact dermatitis), an intravenous injection of the sodium salt of dinitrobenzenesulfonic acid induced complete but only temporary inhibition of an epicutaneous test performed 6 hours later, the original degree of sensitivity being restored after 3 to 5 days. In the present experiments, previously sensitized animals injected with neoarsphenamine intravenously and 6 hours later with neoarsphenamine intradermally remained unresponsive for 28 weeks. It is generally recognized that tolerance will be maintained only so long as enough antigen remains available. Differences in the duration of unresponsiveness obtained with dinitrochlorobenzene compared with that obtained with neoarsphenamine might reflect merely variations in the pharmacokinetics of the haptenes used, but could also be due to fundamental differences in the manner of impairment of the immunological response.

In conclusion, the induction of tolerance of the "Sulzberger-Chase" type is possible in previously sensitized animals, as is the case in the "Felton" and the "protein-overload" type (*1*). It should be noted, however, that the immunologic reaction to neoarsphenamine that we succeeded in repressing is of the tuberculin type, whereas in Chase's "feeding" experiments as well as in the experiments of Coe and Salvin (*10*) and our own performed with dinitrochlorobenzene, we were dealing with a contact dermatitis.

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Reevaluation of Assays Used To Show RNA Induction of Glucose-6-Phosphatase in Ascites Cells

Abstract. *The standard procedures —Glucostat method and Fiske-Subbarow phosphorus determination—are inadequate for the assay of glucose-6-phosphatase activity in Ehrlich ascites cells. Therefore, RNA induction of this enzyme in ascites cells, even if it occurs, cannot be demonstrated by these procedures.*

According to recent reports (*1*, *2*), Ehrlich ascites cells, known to lack glucose-6-phosphatase and tryptophan pyrrolase activities, can be induced to exhibit these activities by treatment with RNA from liver, an organ possessing high levels of these enzymes. Furthermore, the induced tryptophan

pyrrolase activity of RNA-treated cells is reported to be maintained during successive serial transfers in mice (*2*). The importance of these claims appeared to warrant repetition of the original experiments. Preliminary experiments on the RNA-induction of tryptophan pyrrolase activity gave negative results, and we therefore examined induction of glucose-6-phosphatase activity by liver RNA. Our failure to obtain positive results with this enzyme led us to reexamine the adequacy of the two methods used for the assay of its activity, namely, the so-called Glucostat determination of glucose and the determination of phosphate according to Cori and Cori (*3*) which measure respectively the two products of hydrolysis of glucose-6-phosphate by the phosphatase. As shown below, both procedures were found to be inadequate for the quantitative assay of glucose-6-phosphatase activity in Ehrlich ascites cells.

The Glucostat method is based on the following sequence of reactions:

$$\text{Glucose} + O_2 + H_2O \rightarrow H_2O_2 + \text{gluconic acid} \quad (1)$$

$$H_2O_2 + \text{reduced chromogen} \rightarrow \text{oxidized chromogen} \quad (2)$$

These reactions are catalyzed by glucose oxidase and peroxidase, respectively.

Table 1 gives the results of an experiment in which a known small amount of glucose was added to the reaction mixture with or without the usual amount of homogenate of ascites cells. Comparison of the optical densities of tubes 1, 2, and 3 (Table 1) shows that some component of the homogenate—not precipitable by trichloroacetic acid—interferes with the assay; examination of the remainder of the data recorded in Table 1 shows that this interference is due to the inhibition of peroxidase, the mechanism of which has not been elucidated (*4*).

The described inhibition of the Glucostat assay by ascites cell homogenates is not detected unless an internal standard is used in this assay. However, in spite of this precaution, the Glucostat assay cannot be used for the determination of low concentrations of glucose in the presence of ascites-cell extracts. In other words, the method is inadequate for the estimation of glucose-6-phosphatase activity of ascites cells.

The inadequacy of the Fiske-Subbarow procedure (*5*), as modified by Cori and Cori (*3*), for the indicated purposes is revealed by experiments in which known small amounts of phosphate were measured in the presence of the standard concentrations of citrate, glucose-6-phosphate, or both, used in the assay of the phosphatase activity of ascites cells. The results of one such experiment are shown in Table 2. Both citrate and glucose-6-phosphate under the condition of the glucose-6-phosphatase assay, interfere with the determination of orthophosphate by the Fiske-Subbarow procedure (Table 2, compare lines 1, 5, and 6, column 4). Since the inhibitory effect is overcome when the concentration of ammonium molybdate is increased (compare columns 4 and 5), the inhibition appears to be due to the chelation of molybdate by both citrate and glucose-6-phosphate. Thus low concentrations of orthophosphate cannot be measured quantitatively with this method unless the usual amount of molybdate is increased several fold.

The inadequacy of the assay is further illustrated by an experiment in which glucose-6-phosphatase activity of a homogenate of liver cells was measured by the "revised" and the "standard" methods, that is, with two different concentrations of molybdate. With the revised method, 0.05 ml of liver homogenate (about 1.5 mg protein per tube) released 12 μg P per 15 minutes per milligram of protein. With the standard method only approximately 1 percent of this activity was detected.

Thus the standard method is inadequate even with liver cells which have a high glucose-6-phosphatase activity. It is not surprising, therefore, that, using the standard procedure, we were unable to detect glucose-6-phosphatase activity in homogenates of ascites cells, whether RNA-treated or not (in these experiments the amount of protein per tube was approximately the same as that in the experiment with liver cells). Clearly, RNA-induction of glucose-6-phosphatase could not be detected by the standard method unless it resulted in much higher specific activity than that of liver cells.

When the glucose-6-phosphatase activity of ascites cells is examined with the revised technique, it is found that phosphate is slowly released from glucose-6-phosphate. However, the amount of phosphate released at *p*H 5.3 is five times higher than that released at *p*H 6.8. Thus the release of phosphate from glucose-6-phosphate by ascites cell homogenates appears to be due to an acid phosphatase of low specificity.

Table 1. Effect of ascites cell homogenate on peroxidase activity. The reaction mixtures (final volume 4.0 ml) contained 90 μmole of sodium citrate buffer, *p*H 6.8; 2ml of 10 percent trichloroacetic acid; and the indicated amounts of glucose, 0.003 percent H_2O_2, and of ascites cell homogenate, added in the foregoing order. The cell homogenates were prepared by grinding washed ascites cells with 2 volumes of distilled water at 2°C in a ground glass homogenizer for 4 minutes. After centrifugation, a 2-ml sample of the reaction mixture was neutralized with 0.5 ml of 0.5*M* potassium phosphate, *p*H 7.4, and 0.18 ml of 2*N* KOH. The Glucostat reagent (Worthington, 2 ml) was then added, and the reaction mixture was incubated for 30 minutes at 37°C. Thereafter 0.1 ml of 1*N* HCl was added to stabilize the color. Optical densities (O.D.) were measured with a Zeiss spectrophotometer at 400 mμ.

Tube No.	Amt. of substance added			O.D. at 400 mμ
	H_2O_2 (ml)	Glucose (μg)	Homogenate (ml)	
1	0	0	0	0.000
2	0	60	0	.205
3	0	60	0.5	.000
4	0.2	0	0	.016
5	.4	0	0	.026
6	.6	0	0	.048
7	.8	0	0	.062
8	.8	0	0.3	.000

Table 2. Effect of citrate, glucose-6-phosphate (G-6-P), and ammonium molybdate on the determination of orthophosphate. The reaction mixtures consisted of 0.9 ml of 0.1*M* sodium citrate, *p*H 6.8; 0.15 ml of 0.1*M* potassium G-6-P* dissolved in distilled water; and potassium phosphate, as indicated in the table, in a final volume of 3.0 ml. After 10 minutes, 3 ml of 10 percent trichloroacetic acid was added to each tube, the contents of the reaction vessel were well mixed, and two 1.5-ml samples were transferred to clean tubes. Added to each sample were 0.1 ml of 10*N* H_2SO_4, 0.1 or 0.3 ml of 5 percent ammonium molybdate, and 0.1 ml of reducing agent† in a final volume of 2.0 ml. After 10 minutes, the optical density (O.D.) was measured with a Zeiss spectrophotometer at 660 mμ.

Phosphorous (μg)	G-6-P (μmole)	Citrate (μmole)	O.D. at 660 mμ	
			0.1 ml molybdate	0.3 ml molybdate
20	0	0	0.300	0.320
0	15	90	.000	.150
20	15	90	.010	.455
20	15	0	.450	.460
20	0	90	.030	.315
20	60	0	.125	.875

* Obtained from Sigma Chemical Corporation (purity of at least 98 percent). † The reducing agent was prepared by grinding 0.5 g of 1-amino-2-naphthol-4-sulfonic acid with 30 g of $NaHSO_3$ and 1 g of Na_2SO_3. A fresh solution was prepared each day by dissolving 1.56 g of this mixture in 10 ml of water.

This experiment is presented to show that the revised method of phosphorus determination is sensitive enough to measure the very low glucose-6-phosphatase-like activity of ascites cells which is undetected by the standard method. Yet the revised assay did not reveal an increase of phosphorus release from glucose-6-phosphate by ascites cells after treatment with liver RNA (*6*).

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Effect of Bromination on the Biological Activities of Transfer RNA of Escherichia coli

Abstract. *A transfer RNA molecule accepts a particular amino acid from an activating enzyme and subsequently transfers it to the end of a growing peptide chain, in association with ribosomes and messenger RNA. The acceptor and transfer functions of transfer RNA differ in their sensitivity to damage by bromination, and thus appear to comprise different areas on the transfer RNA molecule.*

The biological activities of transfer RNA are recognized to be the acceptance of activated amino acids with the formation of aminoacyl-RNA, and the transfer of amino acid from aminoacyl-RNA to the template on the ribosomes (*1*). The structural specificities in these two functions of transfer RNA have not been established. In reality, each of these functions is in itself multiple. The transfer RNA molecule both recognizes the activating enzyme and accepts from it an activated amino acid by esterification to the —CCA (*2*) end grouping. The transfer step involves positioning of the transfer RNA molecule on the messenger RNA template (presumably by a base pairing operation), correct spatial arrangement of two adjacent transfer RNA molecules before the chain-lengthening step; and nucleophilic attack of the α-amino group on the aminoacyl-RNA adjacent to it. Guanosine triphosphate (GTP) and special enzymes participate in these operations. Our purpose has been to determine whether the two complexes of functions of transfer RNA described —recognition-acceptor and coding-donor—can be distinguished from each other by a chemical agent which damages the transfer RNA molecule in a selective way.

The effect of bromination on the recognition acceptor activities of transfer RNA from yeast and *Escherichia coli* has been reported (*3*, *4*). Dilute aqueous solutions of bromine were added to an equal volume of polynucleotide solution. The detailed mechanism of the addition of a bromonium ion from Br_2 to the nucleic acid bases uracil, cytosine, and, to a lesser extent, guanine has been discussed (*4*). There was no reaction of bromine with adenine. In the case of uracil, 5-bromo-6-hydroxydihydrouracil was the principal product. From a given molecule of bromine, Br_2, a single atom is generally introduced into a nucleic acid base. The Br:nucleotide ratios mentioned hereafter refer to this single bromine atom added from each molecule of Br_2.

These studies indicated a striking species difference in the sensitivity of transfer RNA's to bromination as measured by the capacity of the brominated RNA's to accept amino acids. They also suggested the presence of separate sites for the acceptor and donor functions of transfer RNA. Our current investigations suggest that the formation of aminoacyl-RNA depends on the undisturbed normal secondary and primary structure of a larger proportion of the RNA molecule than is required for the transfer of the same amino acids to a nascent polypeptide chain on the ribosomes.

The effect of bromination on the formation of aminoacyl-RNA was studied. L-Valine-1-C^{14}, DL-phenylalanine-1-C^{14}, and L-lysine-C^{14} (*5*) were diluted to a specific activity of 20, 25, and 5.6×10^6 count/min per micromole, respectively. Bromination of transfer RNA of *E. coli* (*5*), and the formation of aminoacyl-RNA, were carried out as described (*3*, *4*) with the minor modification of generally omitting the nitrogen treatment after bromination since it was considered unnecessary. The effect of bromination on the formation of aminoacyl-RNA, as compared with the same reaction in unbrominated control samples of RNA, is shown in Fig. 1. As described previously, the formation of aminoacyl-RNA is inhibited by bromination of transfer RNA, the extent of inhibition varying according to the amino acid used in the assay. Thus, when *E. coli* transfer RNA was brominated at a ratio of two atoms of bromine to 80 nucleotide residues of RNA, the formation of phenylalanyl-, valyl-, and lysyl-RNA was inhibited by 18, 30, and 58 percent, respectively, and at a higher bromine to nucleotide ratio (8 bromine atoms to 80 nucleotides of RNA), the corresponding percentage inhibitions were 60, 72, and 75, respectively. Kinetic studies indicate that at this time interval (15 minutes) the formation of aminoacyl-RNA has reached a plateau. If random bromination is assumed, the large percentage of inhibition at low bromination cannot be caused only by the direct bromination of a recognition site composed of a small number of nucleotides (a triplet, for example) (*3*) since too few bromine molecules are involved.

On the other hand, the inhibition can be explained in one of the following ways. (i) The secondary structure of a sizeable segment of the molecule may be altered by bromination in a way that may critically disturb the spatial orientation of an adjacent small recognition site. (ii) The recognition site itself may consist of a large fraction of the transfer RNA molecule. (iii) In terms of the dual function of RNA already mentioned, in its recognition-acceptor role it is possible that the recognition area in itself may remain intact, but the acceptor site (the 2′ or 3′ ribosyl hydroxyl group) may become sterically too distant from the aminoacyl-adenylic acid to permit esterification. Further experimentation is required to distinguish these possibilities, and in this connection the recent techniques of Loftfield and Eigner (*6*, *7*) appear to offer promise.

As shown in Table 1, bromination of uracil residues results in weakening of the hydrogen-bonding potential for adenine residues. When approximately equal quantities of polyadenylic acid